EVIDENCE OF CHITOSANASE INVOLVEMENT IN THE PROTECTION OF BACTERIA AGAINST THE ANTIMICROBIAL ACTIVITY OF THE CHITOSAN

by

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FACULTÉ DES SCIENCES
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LA CHITOSANASE EN TANT QUE FACTEUR DE PROTECTION DES BACTÉRIES CONTRE L'ACTIVITÉ ANTIMICROBIENNE DU CHITOSANE

par

Mariana Gabriela Ghinet

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To my wonderful parents, Gheorghe and Sabina, who have raised me to be the person I am today. Despite the huge distance that separates us, you have been with me every step of the way, through good and bad times. To my brother Sebastian, my inspiration and model in life and to my sister- in-law Florenta, who encouraged me throughout the years. To my fiancé, Johny, with whom I have spent the most wonderful years of my life, filled with love and happiness.

I dedicate this Thesis.

SUMMARY

Chitosan, a biopolymer composed of β-(1,4)-linked D-glucosamine and *N*-acetyl-D-glucosamine residues has multiple industrial applications. Recently, chitosan has gained great interest due to its antimicrobial activity. Chitosan has antimicrobial activity against a wide range of target organisms such as bacteria, fungi and viruses. This antimicrobial activity is based on its cationic character, and is mediated by the chitosan's positively charged amino groups interactions with negatively charged residues in the bacterial cell wall. Enzymes with chitosanase activity catalyzing the hydrolysis of glycoside linkages in chitosan are found in many organisms, including bacteria, fungi, and plants. In the last three decades, chitosanases have been intensively studied as tools for biotechnological transformation of chitosan. However, less is known about their physiological functions in chitosanase-producing microorganisms. Previous reports have characterized chitosanases as metabolic enzymes allowing bacteria to use chitosan as carbon and nitrogen sources.

The aim of this research project was to examine chitosanases significance as possible resistance factors against the antimicrobial effect of chitosan. Our work, as well as previous studies realized in our laboratory, showed that expression of a heterologous chitosanase gene in the Gram-negative bacterium *Escherichia coli* (naturally devoid of chitosanase activity) increases the level of resistance against chitosan. Interestingly, the resistance level to chitosan was influenced by the relative activity of the heterologous chitosanase. The expression of inactive heterologous chitosanase did not confer any resistance to chitosan supporting our hypothesis that chitosanases may have a role in the protection against the antimicrobial effect of chitosan.

In order to obtain more direct evidence sustaining our hypothesis, we inactivated the chitosanase gene from *Streptomyces lividans* TK24. Hence, we developed a new system for gene disruption and replacement in *Streptomyces* with cytosine deaminase as negative selection marker. The disruption of the chitosanase gene in *S. lividans* TK24 resulted in an

increased susceptibility of the mutant strain towards the toxic effect of chitosan. Our *in vivo* experiments showed that, in the presence of chitosan, growth of this mutant strain as well as its ability for xylose uptake were impaired compared to the wildtype strain. This represents the first genetical proof for the protective role of a chitosanase against the bactericidal effect of chitosan.

In our quest to discover chitosanases with new characteristics, we determined the biochemical properties of the chitosanase CsnA from *Streptomyces coelicolor* A3(2). Our studies revealed that CsnA was, in many aspects, very similar to the chitosanase CsnN174 from *Streptomyces* sp. N174. An interesting feature of the CsnA is its secretion. The signal peptide of the CsnA has a Tat-dependent motif. The CsnA is the first studied chitosanase to be secreted *via* the Tat pathway. These studies also contributed to a better understanding of the chitosanase secretion.

Evidence concerning the role of chitosanases in the protection of bacteria against the bactericidal effect of chitosan was also brought by the study of cell localization of the exo-β-D-glucosaminidase (CsxA) from *Amycolatopsis orientalis*. CsxA has a carbohydrate-binding module (CBM35) with an unusual affinity. This module appended to CsxA recognizes as substrate glucuronic acid, a component of the Gram-positive bacterie cell wall. Thereby, we analyzed by epifluorescence and confocal microscopy the cellular localization of the CsxA-CBM35 in *Amycolatopsis orientalis* cells grown in the presence of chitosan. The microscopy analysis showed that CsxA is anchored to the bacterial cell wall *via* CBM35's interaction with glucuronic acid. Due to its strategic localization, the exo-β-D-glucosaminidase from *Amycolatopsis orientalis* could also contribute to protection against the antimicrobial effect of chitosan.

The results obtained during this study improved our knowledge concerning the physiological role of chitosanases in bacteria. Independent of their cellular localization, intracellular, cell wall-anchored or secreted, the chitosanases play an important role in bacterial resistance to chitosan. Furthermore, our results contribute to a better understanding of the chitosanase function versatility in bacteria.

SOMMAIRE

Le chitosane, un biopolymère composé de résidus de D-glucosamine et *N*-acétyl-D-glucosamine liés par liaisons β-(1,4), possède des applications industrielles multiples. Récemment le chitosane a reçu beaucoup d'intérêt grâce à son activité antimicrobienne. Le chitosane montre une activité antimicrobienne envers des nombreux microorganismes comme les bactéries, les champignons et les virus. Cette activité antimicrobienne est basée sur son caractère cationique. Grâce à ses groupements aminés chargés positivement, le chitosane interagit avec les résidus chargés négativement qui se trouvent au niveau de la paroi cellulaire bactérienne. Les enzymes possédant une activité chitosanase, catalysant l'hydrolyse des liens glycosidiques dans le chitosane, ont été trouvées chez beaucoup d'organismes, incluant des bactéries, des champignons et des plantes. Au cours des trois dernières décennies, les chitosanases ont été intensivement étudiées comme outils pour la transformation biotechnologique du chitosane. Cependant, leurs fonctions physiologiques chez les micro-organismes qui les produisent sont peu connues. Des études précédentes ont caractérisé les chitosanases en tant qu'enzymes métaboliques permettant aux bactéries d'utiliser le chitosane comme sources de carbone et d'azote.

Le but de ce projet de recherche était d'examiner la signification des chitosanases comme facteurs possibles de résistance contre l'effet antimicrobien du chitosane. Nos travaux, ainsi que d'autres travaux effectués dans ce laboratoire, ont montré que l'expression d'un gène hétérologue de chitosanase dans *Escherichia coli*, une bactérie Gram-négative naturellement dépourvue d'activité chitosanolytique, augmente le niveau de résistance au chitosane. De plus, le niveau de résistance au chitosane est influencé par l'activité relative de la chitosanase hétérologue. L'expression d'une chitosanase hétérologue inactive n'a pas conféré de résistance au chitosane, fait qui confirme notre hypothèse selon laquelle les chitosanases peuvent jouer un rôle dans la protection contre l'effet toxique du chitosane. Afin d'obtenir des preuves directes soutenant ce rôle protecteur des chitosanases contre le chitosane, nous avons inactivé le gène codant pour la chitosanase CsnA de *Streptomyces lividans* TK24. Pour ce faire, nous

avons développé un nouveau système permettant l'interruption génique et la délétion en cadre de lecture pour les streptomycètes, système utilisant le gène de la cytosine désaminase comme marqueur négatif. L'interruption du gène de la chitosanase de *S. lividans* TK24 a eu comme résultat une plus grande sensibilité de la part du mutant envers l'effet toxique du chitosane. Nos expériences réalisées *in vivo* ont prouvé, qu'en présence du chitosane, la croissance du mutant ainsi que sa capacité à utiliser le xylose comme source de carbone ont été altérées en comparaison avec la souche sauvage. Ces résultats représentent les premières preuves génétiques du rôle protecteur d'une chitosanase contre l'effet bactéricide du chitosane.

Au cours de la recherche de chitosanases poseedant de nouvelles caractéristiques, nous avons déterminé les propriétés biochimiques de la chitosanase CsnA de *Streptomyces coelicolor* A3 (2). Nos études ont montré que CsnA est, sur beaucoup d'aspects, très semblable à la chitosanase CsnN174 de *Streptomyces* sp. N174. Une caractéristique intéressante de CsnA est son mode de sécrétion. Le peptide signal de CsnA a un motif Tat-dépendant. CsnA est la première chitosanase étudiée à être sécrétée par la voie de sécrétion Tat-dépendante. Ces études ont contribué à une meilleure compréhension du processus de sécrétion des chitosanases.

De nouvelles preuves concernant le rôle des chitosanases dans la protection des bactéries contre l'effet bactéricide du chitosane ont été apportées également par les études de la localisation cellulaire de l'exo-β-D-glucosaminidase (CsxA) d'*Amycolatopsis orientalis*. CsxA a un module de liaison aux hydrates de carbone (CBM35) caractérisé par une affinité peu commune. Ce module attaché à la CsxA reconnaît comme substrat l'acide glucuronique, un composant de la paroi cellulaire des bactéries Gram-positives. De ce fait, nous avons analysé par microscopie à épifluorescence et microscopie confocale la localisation cellulaire de CsxA-CBM35 chez les cellules d'*Amycolatopsis orientalis* cultivées en présence de chitosane. L'analyse par microscopie a prouvé que CsxA est ancré à la paroi cellulaire bactérienne par l'intermédiaire de CBM35 qui interagit avec l'acide glucuronique. Grâce à cette localisation stratégique, l'exo-β-D-glucosaminidase d'*Amycolatopsis orientalis* pourrait également contribuer à la protection contre l'effet antimicrobien du chitosane.

Les résultats obtenus pendant cette étude ont amélioré nos connaissances sur le rôle physiologique des chitosanases chez les bactéries. Indépendamment de leur localisation cellulaire; intracellulaire, ancré à la paroi cellulaire ou sécrété, les chitosanases jouent un rôle important dans la résistance bactérienne au chitosane. De ce fait, nos résultats ont permis de mieux comprendre la polyvalence des fonctions des chitosanases chez les bactéries.

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LIST OF ABREVIATIONS

Ap:	Ampicillin
bp:	base pair
CBM:	carbohydrate-binding module
CFU:	colony-forming unit
Cm:	chloramphenicol
DA:	degree of acetylation
DDA:	degree of deacetylation
DiBAC4:	bis-(1,3-dibarbituric acid)-trimethine oxanol
DMSO:	dimethyl sulfoxide
DP:	degree of polymerization
EC:	Enzyme Commission
5-FC:	5'-Fluorocytosine
5-FU:	5'-Fluorouracil
GH:	glycoside hydrolase
GlcN:	D-glucosamine
GlcNAc:	*N*-acetyl-D-glucosamine
Hm:	hygromycin
IPTG:	isopropyl-beta-D-thiogalactopyranoside
kb:	kilo base
K_{cat}:	catalytic constant
kDa:	kilo Dalton
Kan:	kanamycin
Km:	Michaelis constant
LB:	Luria-Bertani medium
MAM:	minimal agar medium
MIC:	minimal inhibitory concentrations
Mn:	number average molecular mass

Mw:	molecular weight
neo:	neomycin
NPN:	1-*N*-phenylnaphthylamine
pK_a:	acid dissociation constant
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
RPM:	revolutions per minute
SDS-PAGE:	sodium dodecyl sulfate polyacrylamide gel electrophoresis
S. aureus:	*Staphylococcus aureus*
Tat-pathway:	Twin–arginine translocation pathway
TY:	Tryptone/ yeast extract medium
U:	unit
V_{max}:	maximal velocity
v/v:	volume per volume
w/v:	weight per volume
YME:	yeast/ malt extract medium
X-Gal:	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

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INTRODUCTION

Chitin and chitosan

Chitin is the second most abundant renewable natural resource after cellulose (Deshpande, 1986). This insoluble polymer composed of β-(1,4)-linked *N*-acetyl-D-glucosamine residues is widely distributed in nature particularly in outer skeleton of insects, marine invertebrates, fungi and algae (Muzzarelli, 1977; Ruiz-Herrera and Xoconostle-Cazares, 1995; Cauchie, 2002). The partially *N*-deacetylated derivative of chitin is called chitosan.

Chitosan is a linear polysaccharide composed of randomly distributed β-(1,4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. Chitosan is produced commercially by alkaline deacetylation of chitin. In nature, chitosan occurs in the cell wall of fungi belonging to the genera *Rhizopus*, *Absidia* and *Fusarium* (Alfonso *et al.*, 1995; Gao *et al.*, 1995), in the chlorophycean algae *Chlorella* sp. (Mihara *et al.*, 1961), in the cell wall of *Saccharomyces*

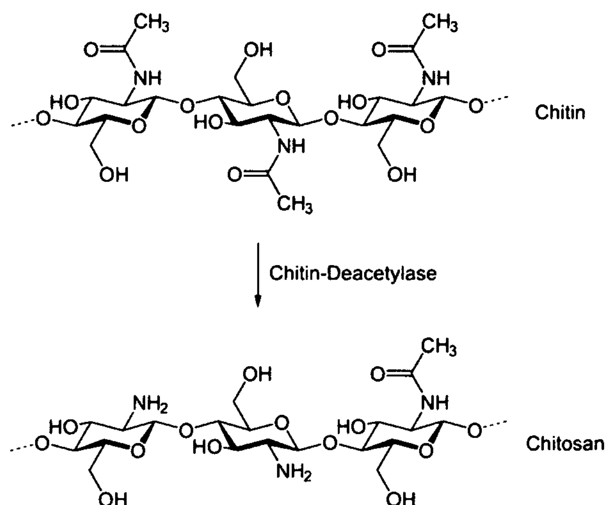


Figure 1: Chitosan synthesis from chitin.

cerevisiae spores (Briza *et al.*, 1988) and transitorily in insects' cuticles (Aruchami *et al.*, 1986). The chitin deacelylase is the key enzyme for chitosan occurrence in nature (Figure1) (Davis and Bartnicki-Garcia, 1984). Compared to cellulose and chitin, chitosan is much less abundant in Nature.

Chitosanase

Generally, chitosanases have been recognized as enzymes that hydrolyze chitosan but not chitin. In 2004, the Enzyme Commission amended the definition of chitosanase as being the enzyme performing endo-hydrolysis of β-(1,4) linkages between D-glucosamine residues in a partially *N*-acetylated chitosan.

Chitosanases (EC 3.2.1.132) were independently discovered by two groups: Monaghan *et al.*, (1972) in a study of the possible use of lytic enzymes to combat pathogenic fungi, and Ramirez-León and Ruiz-Herrera (1972) during a fundamental study of the cell wall architecture.

Chitosanases are members of the glycoside hydrolases group which is divided into 115 families. Based upon their amino acids sequences, chitosanases have been classified into six glycoside hydrolase families: GH5, GH7, GH8, GH46, GH75 and GH80. The families GH5, GH7 and GH8 contain a variety of glycoside hydrolases such as chitosanase, cellulase, licheninase, and endo-1,4-β xylanase while GH46, GH75 and GH80 are currently exclusively composed of chitosanases.

Other enzymes hydrolyzing chitosan

Interestingly, many enzymes with different original specificities have also been reported for their ability to hydrolyze chitosan and its derivatives. In the beginning, most of those double specificities of the enzymes were attributed to contaminations of protein extracts by chitosanases. With time, due to the increased number of reports describing enzymes with specificities for more than one substrate, the term bifunctional enzymes was adopted. These enzymes may be divided into three classes according to their specificity for chitosan hydrolysis.

The first class comprises enzymes with high specificity for chitosan hydrolysis such as: endo-chitinases (EC 3.2.1.14 – GH18 and GH19) (Mitsutomi *et al.*, 1995), exo-β-D-glucosaminidases (also called exo-chitosanases) (EC 3.2.1.165 – GH2 and GH35) (Nanjo *et al.*, 1990) and *N*-acetyl-glucosaminidases (EC 3.2.1.52 – GH3, GH20, GH84) (Muzzarelli, 1993). The members of this class are very important for chitosan assimilation by soil microorganisms. Thus, together with the endo-chitosanases (EC 3.2.1.132), they contribute to the total hydrolysis of the partially *N*-acetylated chitosan into monomers of D-glucosamine and *N*-acetyl D-glucosamine. The endo-chitinases and endo-chitosanases liberate chitooligomers by an endo-type of action (hydrolysis occurs inside of chitosan chain). Then, chitooligomers are further hydrolyzed by exo-β-D-glucosaminidases and *N*-acetyl-glucosaminidases. Those enzymes release a single glucosamine or *N*-acetyl-glucosamine residue from the non-reducing end of the oligomers at a time and the resulting monomers as well as short oligomers may be transported and further metabolized by the bacteria (exo-type of action).

Chitinase

By definition, chitosan is a partly *N*-deacetylated derivative of chitin, so it is not surprising to find bacterial endo-chitinases able to recognize chitosan as substrate and to specifically hydrolyze it. The chitinases degrading chitosan belong to the GH18 and GH19 families, and their hydrolytic action towards chitosan is influenced by the *N*-acetylation degree of this polymer. The endo-chitinases hydrolyze chitosan with high to moderate degree of *N*-acetylation. For instance, previous studies on chitinases A1 and D (GH18) from *Bacillus circulans* WL-12 (Mitsutomi *et al.*, 1995) showed that these chitinases efficiently hydrolyzed the *N*-acetyl-β-D glucosaminidic bonds in 50% *N*-acetylated chitosan. Similarly, the chitinases I (GH18) and II (GH19) from *Burkholderia gladioli* CHB101 (Shimosaka *et al.*, 2001) were efficient at hydrolyzing 30% *N*-acetylated chitosan. Those bacteria also secrete endo-chitosanases which hydrolyze, with maximal efficiency, chitosan with 0-30% *N*-acetylation degree (Mitsutomi *et al.*, 1998; Shimosaka *et al.*, 2000). The contribution of these two types of glycoside hydrolases confers to the bacterial host the capacity to use chitosan with different *N*-acetylation degrees as carbon source.

Exo-β-D-glucosaminidases

In most cases, chitosanases are endo-type enzymes. The exo-β-D-glucosaminidases are also known as exo-chitosanases and have been isolated from few microorganisms such as the fungi *Aspergillus oryzae* IAM2660 (Zhang *et al.*, 2000), *Aspergillus fumigatus* KH-94 (Kim *et al.*, 1998), *Aspergillus flavus* IAM2044 (Ji *et al.*, 2003), *Hypocrea jecorina* (formely *Trichoderma reesei* PC-3-7; Nogawa *et al.*, 1998), *Penicillum funiculosum* KY616 (Matsumura *et al.*, 1999) and the bacterium *Amycolatopsis orientalis* (Nanjo *et al.*, 1990). All these enzymes have been purified and characterized from a biochemical point of view. Based on their amino acid sequence, the exo-β-D-glucosaminidases from *Amycolatopsis orientalis* (Côté *et al.*, 2006) and

Trichoderma reesei PC-3-7 (Ike *et al.*, 2006) belong to the GH2 family, while the exo-β-D-glucosaminidase from *Thermococcus kodakaraensis* KOD1 belongs to GH35 (Tanaka *et al.*, 2003). The culture supernatants of *Aspergillus* and *Amycolatopsis* strains contained endo-chitosanases along with exo-β-D-glucosaminidases, implying the cooperation of these enzymes in chitosan metabolism. The exo-β-D-glucosaminidase from *Amycolatopsis orientalis* is the most studied in terms of structure and function (Côté *et al.*, 2006, Fukamizo *et al.*, 2006; van Bueren *et al.*, 2009). Interestingly, the exo-β-D-glucosaminidase from *Thermococcus kodakaraensis* KOD1 was reported to be involved in a novel chitinolytic pathway (Tanaka *et al.*, 2004). This new chitinolytic pathway is based on the concerted action of the exo-β-D-glucosaminidase and of a diacetylchitobiose deacetylase. The GlcNAc dimers produced from chitin by the chitinase from *Thermococcus kodakaraensis* KOD1 are further *N*-deacetylated by a diacetylchitobiose deacetylase at the non-reducing end. GlcN-GlcNAc dimers are then hydrolyzed by the exo-β-D-glucosaminidase to GlcN and GlcNAc residues which are easily metabolized by bacteria (Tanaka *et al.*, 2004). Moreover, the exo-chitosanases from *Hypocrea jecorina* PC-3-7 and *Aspergillus oryzae* IAM2660 are inducible only by GlcNAc while the one secreted by *A. orientalis* is inducible by chitosan and by GlcN, which suggests that the fungal and bacterial exo-β-D-glucosaminidases may possess different physiological functions.

Cellulase and lysozyme

The second class of enzymes capable of hydrolyzing chitosan includes enzymes with moderate selectivity for chitosan, such as cellulase and lysozyme. Cellulases (EC 3.2.1.4) are enzymes hydrolyzing the β-(1,4)-D-glucosidic linkages in cellulose. Most of the cellulases have a significant hydrolytic activity towards chitosan and some of them may hydrolyze chitosan with an activity almost equivalent to that of chitosanases. This special behavior may be explained by the structural similarity of chitosan and cellulose. Cellulose is a polymer of β-(1,4)-linked D-glucose residues. Thus, chitosan may be considered as an aminated derivative

of cellulose obtained by the replacement of the C-2 hydroxyl group with amino groups (Figure2). Therefore, this capacity of cellulase to hydrolyze chitosan seems to be attributed to a nonspecific recognition of the group at the C-2 position in glucose or glucosamine, when the enzyme-substrate complex is formed. It is known that the specificity of the enzymatic reaction catalyzed by glycoside hydrolases is mostly determined by the characteristics of the substrate binding subsites. Consequently, in order to understand this phenomenon we have to analyze the key amino acids for substrate-binding in chitosanases and cellulases. The chitosanases from the GH46 family recognize partially *N*-acetylated chitosan with high selectivity. In their case, the key amino acids involved in substrate binding are invariably carboxylic acids such as: spartic acid and glutamic acid (Katsumi *et al.*, 2005) which can electrostatically interact with the positive charges of the chitosan chain. In the cellulase case, substrate binding sites are occupied by positively charged amino acids such as histidine, arginine and hydrophobic amino acids such as tryptophan, which may explain the less selective recognition of chitosan by these enzymes (Mulakala and Reilly, 2005).

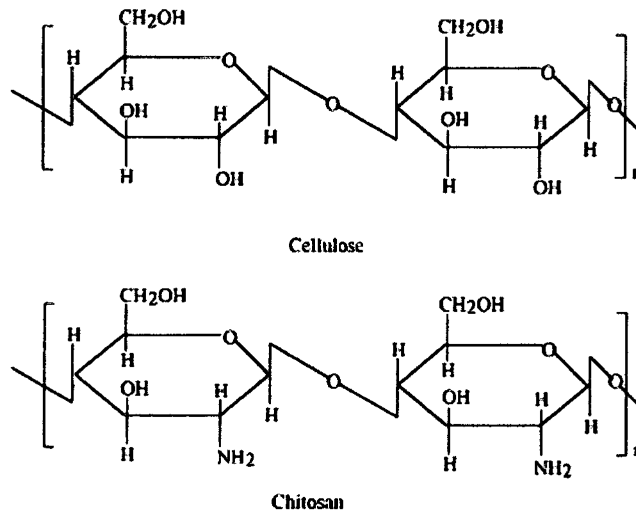


Figure 2: Molecular structure of cellulose and chitosan.

The existence of bifunctional enzymes with cellulase-chitosanase activity is well documented. In many cases, the term bifunctionality may be confusing, when it cames to the classification of the respective enzymes. Depending on the experimental conditions such as the choice of

substrate, temperature, buffer and pH, one type of activity may be favoured to the detriment of the other. For example, the bifunctional cellulase-chitosanase produced by the Gram-negative microorganism *Myxobacter* sp. AL-1 (Pedraza-Reyes and Gutiérrez-Corona, 1997) showed a better chitosanase activity at 70°C than at 42°C, when chitosan hexamer was used as substrate, but a greater cellulase activity at 42°C than at 70°C, when carboxymethyl- cellulose was used as substrate. Under their respective optimal conditions, the chitosanase and cellulase activities were very similar: 61U/mg and 48 U/mg, respectively.

Bifunctional cellulases from various sources show different substrate specificities. The enzymes from *B. cereus* D-11 (Gao *et al.*, 2008), *Myxobacter* sp. AL-1 (Pedraza-Reyes and Gutiérrez-Corona, 1997) and *Bacillus circulans* WL-12 (Mitsutomi *et al.*, 1998) show comparable cellulase and chitosanase activities. Moreover, Cel8A from *Lysobacter* sp. IB-9374 (Ogura *et al.*, 2006) displays a chitosanase activity that is 40% of its cellulase activity. Interestingly, most of the bacterial bifunctional cellulase-chitosanases belong to the GH8 family and are secreted when carboxymethyl-cellulose or glucan is used as carbon source. Moreover, analysis of their primary structure showed high homology with glucanases from GH8 but not with the highly selective chitosanases of the GH46 family.

Another enzyme known to catalyze chitosan hydrolysis with moderate selectivity is lysozyme (EC 3.2.1.17). Ordinarily, lysozyme catalyzes the hydrolysis of 1,4-beta-linkages between *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid residues in peptidoglycans found in the bacterial cell wall. There is a structural resemblance between chitosan and peptidoglycans. The ability of lysozyme to hydrolyze partially *N*-acetylated chitosan is well documented. Thus, it was shown that hen egg white lysozyme and human lysozyme (Sashiwa *et al.*, 1991; Nordtveit *et al.*, 1994) can both hydrolyze soluble chitosan. The *N*-acetylation degree of chitosan is a key parameter influencing the capacity of lysozyme to hydrolyze chitosan. Hence, it was shown that lysozyme is more active against chitosans with a high *N*-acetylation degree. Interestingly, hen egg white lysozyme and GH46 chitosanase share the same structural

organization of the substrate binding and catalytic cleft (Monzingo *et al.*, 1996). Together, this explains the capacity of lysozyme to bind and hydrolyze chitosan. The moderate selectivity is due to the presence of basic and hydrophobic amino acids such as arginine and tryptophan in the substrate binding cleft of lysozyme instead of carboxylic acids characteristic of chitosanase (Maenaka *et al.*, 1994). Thus, the presence of arginine will cause a slight repulsion of the positively charged chitosan, while tryptophan participates to non-polar interactions with the pyranose rings of the substrate.

Finally, the third class is constituted of enzymes hydrolyzing partially *N*-acetylated chitosan with low selectivity. In 2005 Kittur *et al.* isolated a pectinase (EC 3.2.1.15) from *Aspergillus niger* which showed endo- and exo- chitosanase activities towards chitosan. Other enzymes such as a lipase from *Mucor circinelloides* (Struszczyk *et al.*, 2008) and papain (Terbojevich *et al.*, 1996) have been also reported for their ability to hydrolyze chitosan. It is matter of discussion whether or not the chitosanase activities of these enzymes was due to the proteins themselves or to some minor impurities in the preparations.

Occurrence of chitosanases in Nature

Chitosanases are widely distributed in Nature. Since their discovery in 1972, chitosanases have been isolated from numerous prokaryotes, fungi, viruses and plants.

Among prokaryotes, most chitosanase activities have been identified in actinomycetes and bacilli. Actinomycetes are soil-living, Gram-positive bacteria known for their important role in the decomposition of organic matter such as cellulose and chitin. So, it is not surprising that as much as fifteen actinomycete strains such as *Streptomyces* sp. strain 6 (Price and Storck, 1975), *Amycolatopsis orientalis* (formerly known as *Nocardia orientalis*; Sakai *et al.*, 1991),

Streptomyces sp. strain N174 (Boucher *et al.*, 1992), *Kitasatospora* sp. N106 (formerly known as *Nocardioides* sp. N106; Masson *et al.*, 1995), *Amycolatopsis* sp. CsO-2 (formerly known as *Nocardioides* sp.; Okajima *et al.*, 1995; Saito *et al.*, 2009), *Streptomyces griseus* HUT 6037 (Tanabe *et al.*, 2003) and *Microbacterium* sp. OU01 (Sun *et al.*, 2006) have been isolated as chitosanase producers and their enzymes characterized from a biochemical point of view. Other actinomycetes strains such as *S. coelicolor* A3(2) (Bentley *et al.*, 2002), *S. lividans* TK24 (GenBank accession number GQ438786.1), *S. avermitilis* MA-4680 (Ikeda *et al.*, 2003), *S. sclerotialus* (GenBank accession number AB196768.1), *Streptomyces* sp. AM-7161 (Ichinose *et al.*, 2003), and *Renibacterium salmoninarum* ATTC 33209 (GenBank accession number ABY24857) are also known to possess chitosanase activities. The amino acid sequences for all the strains mentioned above are known and, with the exception of the chitosanase from *Streptomyces griseus* HUT 6037 (GH5), they all belong to the GH46 family.

Bacilli are also Gram-positive bacteria. *Bacillus* strains producing enzymes with chitosanase activity are abundantly present in soil. To date, more than thirty five *Bacillus* strains have been identified as high chitosanase producers. *Bacillus megaterium* P1 (Pelletier and Sygusch, 1990), *Bacillus circulans* MH-K1 (Ando *et al.*, 1992), *Bacillus circulans* WL-12 (Mitsutomi *et al.*, 1998), *Bacillus subtilis* strain 168 (Rivas *et al.*, 2000), *Bacillus coagulans* CK108 (Yoon *et al.*, 2002), *Paenibacillus fukuinensis* D2 (Kimoto *et al.*, 2002), *Bacillus* sp. DAU101 (Lee *et al.*, 2006), *Bacillus* sp. strain S65 (Su *et al.*, 2006), *Bacillus thuringiensis* (Lee *et al.*, 2007), and *Bacillus cereus* D-11 (Gao *et al.*, 2008) are just a few of those that have been characterized from a biochemical point of view. In fact, the real number of soil *Bacillus* strains with chitosanase activities is still to be determined. Most of the *Bacillus* chitosanases are regrouped into the GH8 family with a few exceptions whose primary structure has a high homology with most of the actinomycetes chitosanases, thus they belong to GH46 family.

Thus, it is not surprising that the majority of the chitosanases that have been studied extensively in terms of their catalytic features, protein structures and enzymatic mechanisms,

have been isolated from actinomycetes and bacilli (Boucher *et al.*, 1995; Marcotte *et al.*, 1996; Fukamizo *et al.*, 2005; Saito *et al.*, 1999).

Gram-negative bacteria such as *Myxobacter* sp. AL-1 (Pedraza-Reyes and Gutiérrez-Corona, 1997), *Burkholderia gladioli* CHB101 (Shimosaka *et al.*, 2000), *Pseudomonas* sp. A-01 (Ando *et al.*, 2008), *Mitsuaria chitosanitabida* (Amakata *et al.*, 2005) and *Serratia marcescens* TKU011 (Wang *et al.*, 2008) were reported to efficiently produce chitosanases. Moreover, a gene coding for a chitosanase activity was identified in the genome sequence of the cyanobacterium *Nostoc punctiforme* PCC 73102 (GenBank accession number ACC80641.1). The chitosanase from *Myxobacter* sp. AL-1 belongs to the GH5 family, those from *Burkholderia gladioli* CHB101, *Pseudomonas* sp. A-01 and *Nostoc punctiforme* PCC 73102 are members of the GH46 family, while the one from *Mitsuaria chitosanitabida* belongs to GH80.

An interesting group of microorganisms producing chitosanases is represented by fungi. Fungi are known to possess chitosan, the chitosanase's substrate, in their cell wall. The first fungal chitosanase was isolated by the team of Fenton and Eveleigh in 1981. They produced the chitosanase from *Penicillium islandicum* by cultivating this fungus in the presence of *Rhizopus rhizopodiformis* hyphae as substrate. Since then, as much as twenty three chitosanase producing fungal strains have been isolated and enzymes with chitosanase activity were further characterized. *Mucor rouxii* (Alfonso *et al.*, 1992), *Fusarium solani* (Shimosaka *et al.*, 1993), *Aspergillus oryzae* (Zhang *et al.*, 2001), *Aspergillus aculeatus* F50, *Aspergillus fumigatus* (Cheng *et al.*, 2006), *Trichoderma reesei* (Ike *et al.*, 2007), and *Gongronella* sp. JG (Wang *et al.*, 2008) are just some of those chitosanolytic fungal strains. Fungal chitosanases are regrouped in the GH75 and GH7 families, and among them, the one originating from *Fusarium solani* is the best characterized (Shimosaka *et al.*, 1996). The fungal chitosanases have no sequence similarities with the bacterial ones. Interestingly, in some cases, the addition of chitosan to the culture medium inhibited fungal growth, which may suggest that fungal

chitosanases may play a different role than the utilization of the exogenous chitosan as nutrient.

The virus strains' group for which chitosanase activity was detected is less representative, compared to the bacterial one. To date, two CVK2 virus strains infecting *Chlorella viridis* (Yamada *et al.*, 1997) and PBCV-1 infecting *Paramecium bursaria* (Sun *et al.*, 1999; Han *et al.*, 2002), were isolated for their ability to degrade the rigid, chitosan containing, cell wall of the algae from *Chlorella* species. Remarkably, analysis of the viral chitosanases amino acid sequences corresponding to the catalytic segment revealed that these enzymes have a high homology with chitosanases produced by *Streptomyces sp.* N174, *Kitasatospora* sp. N106 and *B. subtilis* (Fukamizo and Brzezinski 1997), therefore, they belong to the GH46 family. The chitosanase from the CVK2 virus strain was purified and a role of this chitosanase in the lytic cycle of the virus was proposed.

Interestingly, enzymes with chitosanase activity have also been isolated from leaf, seed, roots, and fruit extracts of several plant species such as *Cucumis sativus* (cucumber), *Citrus sinensis* (sweet orange), *Nicotiana tabacum* (tobacco) and *Lycopersicon esculentum* (tomato) (El Ouakfaoui and Asselin., 1992a; El Ouakfaoui and Asselin, 1992b; Osswald *et al.*, 1994; Brunner *et al.*, 1998; Pozo *et al.*, 1998). So far, the corresponding enzymes have not been characterized at the primary sequence level.

Therefore, the taxonomical distribution of the chitosanase producers is remarkable. Regarding their primary structure, the chitosanases from different taxonomic groups are well distributed in the six glycoside hydrolase families. The GH7 and GH75 families regroup chitosanases from fungi. The GH8 family is reserved essentially to chitosanases produced by members of the *Bacillus* group. The GH5 and GH46 families are the most diversified from a taxonomical

point of view, as they contain chitosanases originating from actinobacteria, bacilli, Gram-negative bacteria and viruses. Finally, the GH80 family includes only bacterial chitosanases.

Moreover, multiple forms of chitosanases were detected in representatives of all the groups described above. For example, in 1990 Pelletier and Sygusch showed that *B. megaterium* P1 secretes three chitosanases. Later on, other microorganisms in which multiple forms of chitosanases were detected, have been added to the list: *Mucor rouxii* (Alfonso *et al.*, 1992), *Bacillus licheniformis* UTK (Uchida *et al.*, 1992), *Chlorella* virus CVK2 (Yamada *et al.*, 1997), *Streptomyces coelicolor* A3(2) (Bentley *et al.*, 2002), *Aspergillus* sp. CJ22-326 (Chen *et al.*, 2005), and *Microbacterium* sp. (Sun *et al.*, 2006). In plants, multiple chitosanase isoforms were detected as well; up to six chitosanases were seen in the various organs of the plant *Cucumis sativus* (El Ouakfaoui and Asselin, 1992a). In *Citrus sinensis*, four proteins with chitosanase activity were also identified by Osswald *et al.* in 1994. In most cases, these chitosanase activities are encoded by different genes (Alfonso *et al.*, 1992; Bentley *et al.*, 2002) with the exception of *Chlorella* virus CVK2 in which the *vChta-1* gene was shown to produce, by a mechanism of alternate gene expression, two chitosanases with different roles in viral infection (Yamada *et al.*, 1997).

More interesting, some chitosanases have hydrolytic activity on substrates other then chitosan. After examples of chitosanases that are capable of hydrolyzing chitin can be found in the literature. In Nature, chitin is found in the shell of marine invertebrates, insect's cuticles, and in the cell wall of fungi and algae (Muzzarelli, 1977) where it plays an important role in the maintenance of shape or cellular integrity. Following chitin synthesis by chitin synthetase, this polymer is *N*-deacetylated by chitin deacetylase (Davis and Bartnicki-Garcia, 1984) to a certain degree which confers some flexibility to the chitin; flexibility required for example when marine invertebrates or insects are growing, or when the fungal hyphal branches are developing. Therefore, chitosanases from *Enterobacter* sp. G-1 (Yamasaki *et al.*, 1993) and

Mucor rouxii (Alfonso *et al.*, 1992) are able to hydrolyse chitin proccesing a certain degree of *N*-deacetylation.

Chitosanases with cellulase activity have been isolated from various sources such as: *B. cereus* S1 (Kurakake *et al.*, 2000), *B.* sp. KCTC 0377BP (Choi *et al.*, 2004), *Streptomyces griseus* HUT 6037 (Tanabe *et al.*, 2003) and *Trichoderma reesei* (Ike *et al.*, 2007). Interestingly, the chitosanases B and C from *B. megaterium* P1 (Pelletier and Sygusch, 1990) possess comparable enzymatic activities toward chitosan, chitin and cellulose. Even if the structural resemblance between those substrates (polymers of β-(1,4)-linked derivates of glucose) may explain this multiple substrate specificity, the real hydrolysis mechanism needs to be further studied.

Interestingly, for most of the bifunctional cellulase-chitosanases, a single substrate-binding domain is thought to be responsible for interaction with the two substrates, but the mechanism behind this is still unclear. However, Liu and Xia proposed that the bifunctional cellulase-chitosanase isolated from a commercial cellulase preparation, has two different substrate binding domains (Liu and Xi, 2006). However, their binding mechanism and the domain location in the protein are unknown.

Another example of multiple substrate specificity is that of the 40 kDa chitosanase from *Bacillus circulans* WL-12 (Mitsutomi *et al.*, 1998). Substrate specificity analysis revealed that this chitosanase hydrolyzed chitosan (30% *N*-acetylated) and lichenan (β-1,3-1,4-glucan) with similar efficiency. Interestingly, eight years before, this enzyme has been reported as a β-1,3-1,4-glucanase by Bueno *et al.* (1990). Therefore, in order to determine which was the major substrate for this enzyme, Mitsutomi and coworkers analysed the production of this protein by *B. circulans* WL-12 in media supplemented with chitosan and lichenan. The study showed that production of this enzyme was induced by chitosan (30% *N*-acetylated) but not by lichenan.

Thus, this enzyme is first a chitosanase and, it according to its primary structure, has been included into the GH8 family. As most of the chitosanases belonging to the GH8 family, this chitosanase/β-1,3-1,4-glucanase has also the capacity to hydrolyze CM-cellulose (β-(1,4)-glucan), which may indicate that in lichenan the enzyme hydrolyzes only β-(1,4)-linkages.

The fact that chitosanases show different specificities for substrates made Schindler *et al.* hypothesize that: "Evolutionary changes in substrate structure may have influenced the development of the active site of lysozyme so that it could function most efficiently with the particular natural substrate encountered by each species" (Schindler *et al.*, 1977).

Chitosanases: mode of action

Based on the observations presented in the previous sections, two questions may be raised: are there any differences among the chitosanases produced by organisms belonging to the groups described above? And why does microorganism need to secrete more than one chitosanase when the availability of chitosan in soil as nutrient is not even comparable with other carbon sources such as cellulose and chitin? The answer comes from the characteristics of chitosan itself.

An important property of chitosan is its degree of *N*-acetylation. Chitosan wholly *N*-deacetylated or with different *N*-deacetylation degrees (DDA) can be obtained from chitin by various, well controlled chemical methods (Muzzarelli, 1985; Vårum and Smidsrød, 2004). Fully *N*-deacetylated chitosan is not found in nature (Bartnicki-Garcia, 1968). Natural chitosan has a DDA varying from 55 to 95%. The chitosan present in the cell wall of fungi from different genera is characterized by various degrees of *N*-deacetylation: *Absidia coerulea* and *Saccharomyces cerevisiae* 95% DDA, *Mortierella isabelina*, *Aspergillus niger* and *Candida*

albicans 84% DDA, *Fusarium oxysporum* 83% DDA, *Penicillium citrinum* 79% DDA, *Rhizopus oryzae* 90-78% DDA, *Cunninghamella blakesleeana* 65%, and *Mucor rouxii* 95-55% DDA (Briza *et al.*, 1988; Miyoshi *et al.*, 1992; Pochanavanich and Suntornsuk, 2002). These natural chitosans are probably synthesized by the sequential action of chitin synthetase and chitin deacetylase, as shown for *Mucor rouxii* and *Colletotrichum lindemuthianum* (Davis and Bartnicki-Garcia, 1984). Hence, there is diversity in the substrate available for the microorganisms living in soil.

Consequently, the microorganisms adapted to this substrate diversity by producing chitosanases with different substrate specificity. The effect of DDA on chitosanase activity has been well documented (Table1).

Table 1: Substrate specificity of chitosanases from different sources

Source	Substrate specificity (% DDA)	Reference
Streptomyces N174	40 - 99	Boucher *et al.*, 1992 Masson *et al.*, 1994
Amycolatopsis orientalis	59 - 100	Sakai *et al.*, 1991
Amycolatopsis sp. CsO-2	70 - 100	Okajima *et al.*, 1994
Microbacterium sp. OU01 Chitosanase N Chitosanase X	 95 -100 86 -100	Sun *et al.*, 2006
Bacillus sp. No. 7-M	99	Izume *et al.*, 1992
Bacillus circulans	40 -100	Davis and Eveleigh, 1984
Acinetobacter sp. CHB101 Chitosanase I Chitosanase II	 70 - 90 70	Shimosaka *et al.*, 1995
Pseudomonas sp. H-14	62 -100	Yoshihara *et al.*, 1992
Enterobacter sp. G-1	80	Yamasaki *et al.*, 1993
Fusarium solani f. sp.	70 -100	Shimosaka *et al.*, 1993
Mucor rouxii	88 - 98	Alfonso *et al.*, 1992
Citrus sinensis	80 -100	Osswald *et al.*, 1994

The chitosanases from *Bacillus* sp. No. 7-M (Izume *et al.*, 1992), *Pseudomonas* sp. H-14 (Yoshihara *et al.*, 1992), *Amycolatopsis* sp. CsO-2 (Okajima *et al.*, 1994) and *Bacillus* sp.

PI-7S (Seino *et al.*, 1991) showed a preference for chitosan that is approximately 100% *N*-deacetylated. Chitosanases from *Penicillium islandicum* (Fenton and Eveleigh, 1981) and *Streptomyces* sp. N174 (Boucher *et al.*, 1992) could depolymerise chitosan substrates having a wide range of *N*-deacetylation degrees: 40-70% and 79-99%, respectively.

Interestingly, chitosanases from *Fusarium solani* (Shimosaka *et al.*, 1993) and *Amycolatopsis orientalis* (Sakai *et al.*, 1991) can act optimally only on chitosan having 70% *N*-deacetylation whereas that from *Enterobacter* sp. G-1 (Yamasaki *et al.*, 1993) has its best activity on chitosan having 80% *N*-deacetylation. This special behaviour is an indication that the *N*-acetyl glucosamine residues are important in the recognition and reaction mechanism of enzymes on various substrates.

Cleavage specificity

Partially *N*-acetylated chitosan contains four types of linkage between its subunits: GlcN-GlcN, GlcNAc-GlcN, GlcN-GlcNAc and GlcNAc-GlcNAc. The proportion of these linkages varies in function of the degree of *N*-acetylation and their distribution is random in the polymer structure. According to their cleavage specificity, chitosanases are divided into three subclasses. Enzymes of subclass I cleave GlcN-GlcN and GlcNAc-GlcN linkages and includes chitosanases from *Bacillus pumilus* (Fukamizo *et al.*, 1994), *Penicillium islandicum* (Fenton and Eveleigh, 1981) and *Streptomyces* sp. N174 (Fukamizo *et al.*, 1995). Subclass II contains chitosanases from *Bacillus* sp. *No 7-M* (Izume *et al.*, 1992) *Pseudomonas* sp. H-14 (Yoshihara *et al.*, 1992), *Amycolatopsis* sp. CsO-2 (Okajima *et al.*, 1994) and *Bacillus* sp. PI-7S (Seino *et al.*, 1991) that recognize only GlcN-GlcN linkages. Subclass III includes enzymes from *Bacillus circulans* MH-KI (Saito *et al.*, 1999), *Nocardia orientalis* (Sakai *et al.*, 1991), and *Bacillus circulans WL-12* (Mitsutomi *et al.*, 1998) which recognize GlcN-GlcN as well as GlcN-GlcNAc linkages. Thus, analysis of cleavage specificity of the partial *N*-acetylated

chitosan made possible the clear differentiation between chitosanases and chitinases. To date, all studied chitosanases have a high specificity for the cleavage of GlcN-GlcN linkages. In contrast, the chitinases cannot cleave the linkage between two glucosamine residues as they require at least one GlcNAc residue in the cleaved linkage (Fukamizo *et al.*, 1994).

This classification was changed this year by the addition of a fourth subclass following the discovery of an enzyme from the fungus *Alternaria alternata* that specifically cleaves only the GlcNAc-GlcN linkage (Kohlhoff *et al.*, 2009). Due to its new cleavage specificity, this enzyme was called a chitinosanase. This is half a chitinase and half a chitosanase enzyme which cannot be considered a chitinase because it does not cleave GlcNAc-GlcNAc linkages nor a chitosanase because it does not cleave GlcN-GlcN linkages.

Chitosanase secretion

The targeting and transport of proteins to and across biological membranes is a very important characteristic of cellular life. Interestingly, it is thought that 25-30% of the genes coding for proteins from the model Gram-negative organism *E. coli*, or the Gram-positive organism *Streptomyces coelicolor*, encode proteins that will be located either partially or completely outside the cytoplasm. In prokaryotes, two major pathways exist for the export of proteins across the cytoplasmic membrane the Sec pathway and the Tat pathway.

The Sec pathway is the general *Sec*retion route for proteins in most bacteria. This pathway is generally responsible for the transport of newly synthesized proteins out of the cytosol in an unfolded state; thereby they fold into their native structure once released from the membrane. The Sec secretion machinery (also called Sec-translocase) is present in the cytoplasmic membrane of all bacteria, archaea, the thylakoid membrane of plant chloroplasts and the

endoplasmic reticulum of eukaryotes. In bacteria, the Sec pathway is responsible for the secretion of most extracellular proteins with diverse functions in metabolism, substrate uptake and excretion, cell envelope structure, sensing and cell communication (Lee and Schneewind, 2001, Natale *et al.*, 2008).

The Twin–arginine translocation pathway, also called Tat pathway, is involved in the translocation of proteins in their folded state. Tat-translocation systems have been first identified in thylakoid membranes of plant chloroplasts. Functional Tat secretion systems have also been found in the cytoplasmic membranes of many bacteria and archaea (Müller *et al.*, 2005). In bacteria, proteins secreted by this system are found to be involved in energy metabolism, quorum sensing and motility, cell division and biogenesis of the cell envelope, symbiosis and pathogenesis (Berks *et a*l., 2005, Stevenson *et al.*, 2007). In plant chloroplasts, the Tat pathway is involved in the assembly of the oxygen-evolving complex and the cytochrome b_6f complex within the thylakoid membrane (Mould and Robinson, 1991; Mølik *et al.*, 2001).

Since the discovery of the Tat secretion pathway in 1996 by Berks, numerous studies were dedicated to the analysis of the distribution and utilization of this system by organisms of all Kingdoms. Lately, specialized programs such as TATFIND 1.2 (Dilks *et al.*, 2003) and TATscan (Li *et al.*, 2005) were developed for the computational prediction of Tat dependent proteins. In bacteria, most of the secreted proteins are translocated *via* the general Sec pathway, whereas only a subset of bacterial proteins is exported *via* the Tat-pathway. For example, genomic data combined with computational predictions showed that for microorganisms such as *Rickettsia prowazekii* and *Staphylococcus aureus* only one and two Tat substrates have been identified, respectively (Dilks *et al.*, 2003). Moreover, *Fusobacterium nucleatum* and *Lactococcus lactis* lack a Tat system. Interestingly, in other bacteria such as *Caulobacter crescentus*, *Sinorhizobium meliloti* and *Streptomyces coelicolor* this pathway seems to be more extensively used since 88, 94, and 129 putative Tat substrates

have been identified, respectively (Dilks *et al.*, 2003; Li *et al.*, 2005). Moreover, it was shown that in chloroplasts, the number of Tat substrates is equivalent to that of Sec substrates (Müller *et al.*, 2005). Thus, even if the Sec pathway is the general secretion pathway in the majority of organisms, it seems that the degree to which the Tat pathway is used is quite variable, even among related organisms.

Secreted proteins are synthesized in the cytoplasm in a precursor form with an N-terminal extension named signal peptide. This signal sequence is required for correct targeting of the protein to the secretion systems on the cytoplasmic site of the membrane and is cleaved after secretion by a specialized protease. The signal peptide sequence has a tripartite structure: a positively charged amino-terminal (n-region), a hydrophobic core (h-region) and a polar carboxyl terminal (c-region) region.

There are several significant differences between Sec- and Tat-specific signal peptides (Figure 3). Tat signal sequences are on average longer than Sec signals, due to larger n-regions (5-24 residues compared to 1-5 residues in Sec signal peptides). The h-region of Tat signals comprises 12–20 uncharged residues thus being slightly longer and less hydrophobic than h-regions of Sec-substrates (7-15 residues). Furthermore, Tat signal sequences are characterized by a conserved pattern of amino acids which includes two important arginine residues, the twin-arginine motif, located at the junction between the n- and h-regions. In bacteria, this motif has been described as Z-R-R-x-φ-φ, where Z stands for any polar residue and φ for hydrophobic residues.

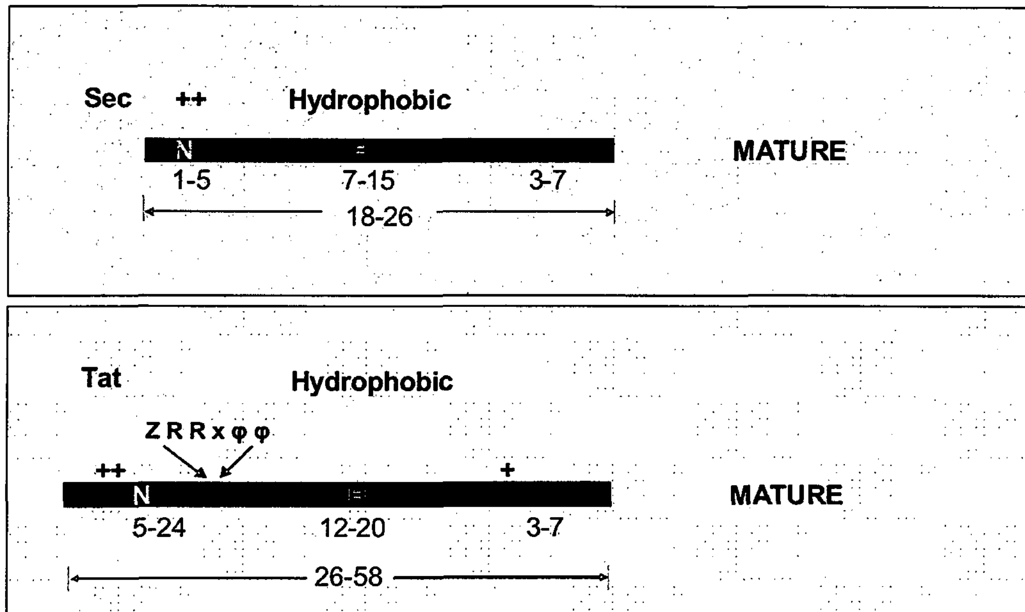
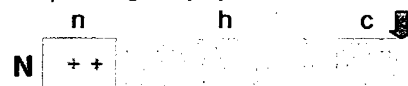


Figure 3: Comparison of the tripartite structure of Sec and Tat signal peptide sequence.

This Tat motif is extremely well conserved and is crucial for the role of Tat signal peptides (Figure 4). Genetic studies showed that replacement of the Arg-Arg pair with a Lys-Lys motif completely abolished Tat-specific export, while substitution of a single Arg by Lys affected only the rate of translocation *via* the Tat system (DeLisa *et al.*, 2002). Tat-specific c-regions frequently contain a positively charged amino acid residue (+ Figure 3), which was proposed to function as a "Sec-avoidance signal" (Bogsch *et al.*, 1997) and a proline residue acting as a 'helix breaker' to facilitate peptidase recognition of the cleavage site. The signal peptidase recognition site is conserved in Sec- and Tat-dependent peptides and is represented by an A-X-A motif found at end of the c-region.

Generic signal peptide structure



Twin-arginine signal peptides

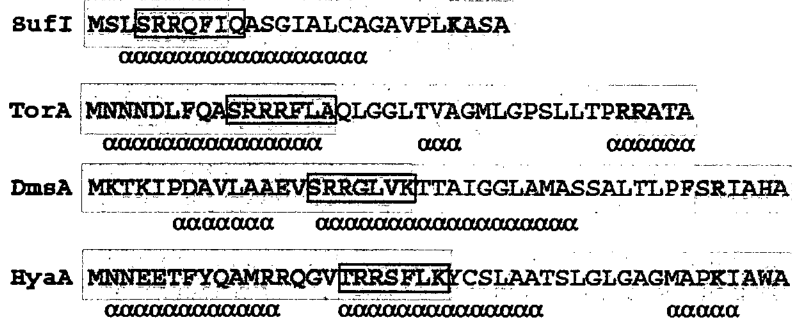


Figure 4: Representation of Tat signal peptides with the conserved residues forming the twin-arginine motif (boxed). (From Palmer *et al.*, 2005, modified). n-region is represented in green, h-region in orange and c-region in purple. The "Sec-avoidance motif", represented by the highly conserved positively charged amino acids found within the c-regions, is shown in bold. Amino acid sequences of twin-arginine signal peptides from *E. coli* DmsA (dimethyl sulfoxidereductase); SufI (copper oxidase); TorA (trimethylamine N-oxide (TMAO) reductase) and HyaA (hydrogenase).

This motif is recognized by the type I signal peptidase, a membrane-bound enzyme that cleaves the signal sequence from the mature secreted protein domain during or shortly after translocation. Interestingly, when the A-X-A site of a Sec-dependent signal sequence was mutated, no protein secretion was observed (Pagé *et al.*, 1996), suggesting the importance of this conserved motif for the maturation of proteins translocated *via* the Sec pathway. In contrast, mutations of the A-X-A signal peptidase recognition site of the Tat-dependent signal peptide had no effect on the precursor processing rate (Li *et al.*, 2006, Sambasivarao *et al.*, 2000). These observations suggest that there is probably a major difference in the processing of the Sec- and Tat-dependent precursors and that processing is probably not carried out by the same signal peptidase I.

It has been shown that the Sec and Tat pathways are also involved in translocation and insertion of membrane proteins into the cytoplasmic membrane. In the case of the Sec-pathway, membrane proteins do not have cleavable N-terminal signal peptides. The integration into the cytoplasmic membrane of those proteins may occur via the Sec-dependent or a Sec-independent pathway, both mechanisms involving the YidC protein (Samuelson *et al.*, 2000). Interestingly, in both bacteria and chloroplasts, Tat-targeted integral membrane proteins are synthesized with an N-terminal signal peptide and contain single internal or C-terminal transmembrane helices. Furthermore, integration of the transmembrane helices into the lipid bilayer is strictly Tat-dependent (Hatzixanthis *et al.*, 2003).

Most of the bacterial and fungal chitosanases are secreted extracellularly (Fenton and Eveleigh, 1981; Pelletier and Sygusch, 1990; Boucher *et al.*, 1992; Yamasaki *et al.*, 1993; Okajima *et al.*, 1994; Wang *et al.*, 2008). Little is known of the specific pathway responsible for the chitosanase secretion. Computational analysis of the signal peptide sequences showed that most chitosanases (for which the amino acid sequence had been published) present a Sec-dependent signal peptide and very few had a Tat-dependent signal peptide (R. Brzezinski unpublished data). Among the Tat-dependent chitosanases, five are from *Streptomycetes* strains: SCO0677 from *Streptomyces coelicolor* A3(2), CsnA from *Streptomyces lividans* TK24, SAV2015 from *Streptomyces avermitilis* MA-4680, SAMR0713 from *Streptomyces ambofaciens* ATCC 23877 (all belonging to GH46) and ChoII from *Streptomyces griseus* HUT 6037 (GH5). Three other Tat-dependent chitosanases are from β-proteobacteria strains: *Herbaspirillum* sp. 9, *Mitsuaria chitosanitabida* 3001 and *Mitsuaria* sp. 67 (GH80). To date, only the chitosanase SCO0677 from *Streptomyces coelicolor* was confirmed to be a real Tat-dependent protein by genetical and biochemical studies (Li *et al.*, 2005). Interestingly, SCO0677 is very similar at the molecular level to the chitosanase from *Streptomyces* sp. N174 a Sec-dependent chitosanase, as shown by the unrooted phylogenetic tree (Figure 5) generated following alignment of amino acid sequences of all the chitosanases belonging to the GH46 family (Annexe I).

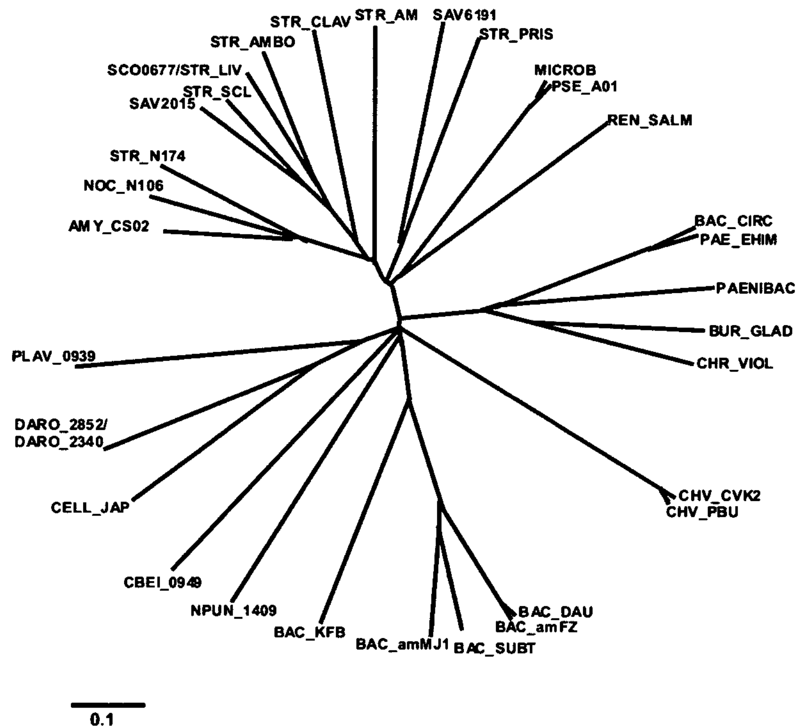


Figure 5: Phylogenetic analysis of the primary sequence of GH46 family members. The amino acid sequences of the respective proteins have been extracted from the following database entry. STR_N174 - *Streptomyces* N174 (P33665); NOC_N106 - *Kitasatospora* sp. N106 (P48846); AMY_CS02 - *Amycolatopsis* sp. CsO-2 (Q9LBG4); BAC_amMJ1 - *Bacillus amyloliquefaciens* MJ-1 (Q0PVM7); BAC_CIRC - *Bacillus circulans* MH-K1 (P33673); BAC_KFB - *Bacillus* sp. KFB-CO4 (Q9RC18); BAC_SUBT - *Bacillus subtilis* subsp. subtilis str. 168 (O07921); BUR_GLAD - *Burkholderia gladioli* CHB101 (Q9XDS6); SAV2015 - *Streptomyces avermitilis* MA-4680 (BAC69726.1); SAV6191 - *Streptomyces avermitilis* MA-4680 (BAC73902.1); SCO0677 - *Streptomyces coelicolor* A3(2) (Q9RJ88); PSE_A01 - *Pseudomonas* sp. A-01 (Q8KZM5); STR_AM - *Streptomyces* sp. AM-7161 (Q7WT07); CHR_VIOL - *Chromobacterium violaceum* ATCC 12472 (AAQ61593.1); PAENIBAC - *Paenibacillus* sp. BH-2005 (Q2PWA1); PAE_EHIM - *Paenibacillus ehimensis* EAG1 (O24825); MICROB - *Microbacterium* sp. OU01 (A7KBW5); CHV_CVK2 - *Chlorella virus* CVK2 (O12288); CHV_PBU - *Paramecium bursaria Chlorella* virus 1 (Q84608); REN_SALM - *Renibacterium salmoninarum* ATCC 33209 (A9WUI6); STR_PRIS - *Streptomyces pristinaespiralis* ATCC 25486 (NW_002063180.1); STR_CLAV - *Streptomyces clavuligerus* ATCC 27064 (NW_002063046.1); STR_AMBO - *Streptomyces ambofaciens* ATCC 23877 (A0AD68); STR_LIV - *Streptomyces lividans* TK24; STR_SCL - *Streptomyces sclerotialus* ISP 5269 (B1Q2K4); PLAV_0939 - *Parvibaculum lavamentivorans* DS-1 (A7HRM9); DARO_2852 - *Dechloromonas aromatica* RCB (Q47C49); DARO_2340 - *Dechloromonas aromatica* RCB (NC_007298.1); CELL_JAP - *Cellvibrio japonicus* Ueda107 (B3PI04); CBEI_0949 *Clostridium beijerinckii* NCIMB 8052 (A6LS03); NPUN_R2009 *Nostoc punctiforme* PCC 73102 (B2J4Y1); BAC_amFZ - *Bacillus amyloliquefaciens* FZB42 (A7Z8H9); BAC_DAU - *Bacillus* sp. DAU101 (A0EQW7).

As shown in Figure 5, the thirty four chitosanases from the GH46 family define four different clusters. The first cluster (in red) is composed mostly of chitosanases produced by bacteria belonging to the Actinomyces genus. Inside this cluster, there are two interesting subclusters: the first is formed by the chitosanases from *Streptomyces* sp. N174 (Boucher *et al.*, 1992) which is the most studied chitosanase for its structure and function and which is very similar to the CsnN106 from *Kitasatospora* sp. N106 (Masson *et al.*, 1995), and the chitosanase from *Amycolatopsis* sp. CsO-2 (Saito *et al.*, 2009). The second subcluster, or the Tat subcluster is composed of SCO0677 from *S. coelicolor*, CsnA from *S. lividans* TK 24, SAV2015 from *Streptomyces avermitilis* MA-4680, SAMR0713 from *S. ambofaciens* ATCC 23877 and the chitosanase from *Streptomyces sclerotialus* ISP 5269 which is the only member of this subcluster that does not have a typical Tat-dependent signal peptide. The second cluster (in green) and the forth cluster (in blue) reunite chitosanases from *Bacillus* species and few Gram-negative bacteria. In between, there is the cluster (in violet) formed by the chitosanases produced by the virus strains *Chlorella* virus CVK2 and *Paramecium bursaria Chlorella* virus 1.

There are also intracellular chitosanases. They have been found in plants (El Ouakfaoui and Asselin, 1992a,b; Osswald *et al.*, 1994), in fungi such as *Mucor rouxii* (Alfonso *et al.*, 1992) and *Mucor cirnelloides* (Struszczyk *et al.*, 2008); and in *Chlorella* virus CVK2 (Yamada *et al.*, 1997). This different cellular localization of chitosanases suggests that they may have different biological roles.

Biological roles of chitosanases

The main function of the microbial extracellular hydrolases is without any doubt the release of nutrients from various substrates, in order to sustain microbial life. In this context,

chitosanases may be used for a metabolic purpose by microorganisms growing in an environment with chitosan or chitin containing substrates.

In Nature, chitosan is found mostly in the cell wall of zygomycetes and other species of fungi (Alfonso *et al.*, 1995; Gao *et al.*, 1995). Studies dedicated to the analysis of natural chitosan showed that the degree of *N*-acetylation of this polymer varies from one strain to another (Briza *et al.*, 1988; Miyoshi *et al.*, 1992; Pochanavanich and Suntornsuk, 2002). In response to the presence in soil of chitosan with different degrees of *N*-acetylation, soil microorganisms had to adapt. Thereby, they express chitosanases that preferentially hydrolyze different kinds of linkages in chitosan (GlcN-GlcN/ GlcNAc-GlcN/ GlcN-GlcNAc). This allows microorganisms to better recycle fungal cell walls and to use the chitosan as carbon and nitrogen sources (Fenton and Eveleigh, 1981; Boucher *et al.*, 1992; Okajima *et al.*, 1994).

In the meantime, many fungal species with extracellular and/or intracellular chitosanase activities have been characterized. For most species, chitosanase production was induced by chitosan, implying a metabolic role for these hydrolases. Interestingly, it has been shown that growth of chitosanase-producing fungi such as *Fusarium solani* and *Aspergillus fumigatus* (Shimosaka *et al.*, 1993, Cheng *et al.*, 2006) was strongly inhibited by addition of chitosan into the culture medium. This may indicate that the main function of these secreted chitosanases does not consist in the degradation and utilization of exogenous chitosan, but they are rather involved in cell division and autolysis. This physiological role had also been proposed for the intracellular chitosanases from *Mucor rouxii* (Alfonso *et al.*, 1992). Moreover, intracellular enzymes with endo-chitosanase, endo- and exo-chitinase and chitinase deacetylase activities were isolated from this fungus. This battery of enzymes contributes to cell wall transformation during the fungal life cycle, from the initiation of hyphal branches, cell separation during growth and to cell wall degradation of the aged mycelium (Alfonso *et al.*, 1992).

Another interesting example of a non-metabolic function of chitosanases is provided by the biology of some viruses such as *Chlorella* virus: CVK2 (Yamada *et al.*, 1997) and the *Paramecium bursaria Chlorella* virus PBCV-1(Sun *et al.*, 1999), as they express chitosanases in order to infect their host. In *Chlorella* virus CVK2 (Yamada *et al.*, 2006) the gene *vChta*1 encodes for two chitosanases with different and well defined roles in virus replication. The larger chitosanase (65 kDa) is packaged in the virion and probably helps in the first steps of the infection by digesting, to a certain degree, the *Chlorella*'s chitosan-containing cell wall. Some other enzymes secreted by the virus at this stage also contribute to host infection. This action is very well synchronized and very precise. *Chlorella*'s cell wall is not disrupted at this stage, thus virus replication can occur inside the alga. Later, the second chitosanase (37 kDa) which remains in the host cytoplasm during the viral replication is thought to participate, together with other viral enzymes, in cell wall digestion during viral release. The sequence of the chitosanase from *Paramecium bursaria Chlorella* virus PBCV-1 is highly homologous with that of 37 kDa chitosanase from *Chlorella* virus CVK2, thus their function may be very similar.

Another well documented role for chitosanases is an antifungal one. Some of the bacterial strains with chitosanase activities were first isolated as antagonists of plant pathogenic fungi. The chitosanase from *Bacillus cereus* D-11 inhibited the growth of *Rhizoctonia solanii,* a pathogen of cabbage, cauliflower and related plants (Gao *et al.*, 2008). Furthermore, following incubation of *Rhizopus oryzae* with the chitosanase from *Amycolatopsis* CsO-2, the fungal hyphae were misshapen rather than forming sporangia and the fungal growth was drastically inhibited (Saito *et al.*, 2009). Another interesting example of this antifungal activity is that of the mycoparasite *Piptocephalis virginiana* which secretes enzymes with chitosanase and chitinase activities in order to degrade the cell wall of its future hosts, fungi from the genus *Mucorales* (Balasubramanian and Manocha, 1986).

In soil, nutriment availability is somehow limited for the inhabiting microorganisms. In order to better adapt to these living conditions, an interesting collaboration between protein production (such as chitosanases and chitinases) and secondary metabolism (antibiotics production) may be observed with soil bacteria. For instance, members of the *Actinomycetes* group such as *Streptomyces griseus* and *Amycolatopsis orientalis* produce chitosanase and chitinases, known for their antifungal activities, as well as antibiotics such as streptomycin (*Streptomyces griseus*; Tanabe *et al.*, 2003) and vancomycin (*Amycolatopsis orientalis*; Nanjo *et al.*, 1990) with antibacterial activity. Thereby, this cooperation between chitosanase/chitinase and antibiotics production may have a role in the maintenance of the microbial balance in the ecological niche where those bacteria are growing. Secreation of chitosanases/chitinases confers an advantage in the utilization of chitosan and chitin from different sources (dead insects, fungi) as carbon source. Furthermore, antibiotic secretion inhibits the sensitive microorganisms to grow, microorganisms which are normally competing for the same nutrients present in soil.

In plants, chitosanases are considered to be secreted as pathogenesis related proteins, involved in the defence mechanism against pathogenic fungi. Thus, chitosanase activities were detected in various plant species following fungal infection (Cuero *et al.*, 1992, Pegg and Young, 1982). Most of the chitosanases produced by plants have also chitinase activity. This confers a great advantage to the plant defensive mechanism. Hence, a wider variety of fungi and even insects may be attacked (Osswald *et al.*, 1994).

In conclusion, chitosanases are involved in various functions such as nutritional, morphogenetic or defensive functions in different organisms. Every year, numerous articles are published regarding new chitosanolytic enzymes, their characteristics and biological functions.

Mechanism of the antimicrobial effect of chitosan

In the last two decades, chitosan and its derivatives have received much attention as antimicrobial agents against bacteria and fungi. Since the first studies describing the antibacterial effect of chitosan (Uchida, 1989) or its antifungal properties (Kendra and Hadwinger; 1984) many potential applications based on this special character of chitosan have been proposed. Chitosan is biodegradable and not toxic for humans (Muzzarelli, 2000). Therefore, the fungicidal activity of chitosan has found many applications in food preservation (Roller and Covill., 1999; Rhoades and Roller, 2002). Many studies proposed that the antibacterial properties of this biopolymer can be exploited in the textile industry, for antimicrobial-finished textiles (Takai *et al.*, 2002), and in pharmacology, in the fabrication of wound dressing (Ueno *et al.*, 2001). Interestingly, various studies also showed that chitosan is an elicitor of plant defense mechanisms (reviewed by Rabea *et al.*, 2003). Moreover, treatment of plants with chitosan inhibits virus infection by stopping viral replication at late stages (Pospieszny *et al.*, 1991).

In recent years, many studies were dedicated to the antimicrobial activity of chitosan and showed that degree of *N*-deacetylation and of polymerization are key factors for this function (Liu *et al.*, 2001; Park *et al.*, 2004). Furthermore, it has been shown that the effectiveness of chitosan depends also on the tested concentration, on environmental conditions, and on the type of microorganism. Therefore, the studies concerning the antimicrobial effect of chitosan may be divided into two categories: 1) Studies realized at lethal doses which allowed determining the bactericidal and fungicidal role of chitosan and 2) studies realized at sub lethal doses of chitosan where the polymer was perceived as a stress agent by the microorganisms.

The first category of studies is based on the determination of the minimal inhibitory concentration of chitosan (minimal concentration of chitosan at which no bacterial or fungal growth can be observed). The minimal inhibitory concentrations (MICs) reported for specific

target organisms are influenced by a multitude of factors such as pH and composition of the growth medium, degree of polymerization and of *N*-acetylation of the chitosan.

It is generally accepted that the antimicrobial functional group in chitosan is the free amino positively charged (NH_3^+) group of glucosamine residues. The inhibitory activity of chitosan is higher at pHs lower than 6 (pK_a value of chitosan is 6.2), when most amino groups are in the free base form. Studies using chitosan with different degrees of *N*-deacetylation (DDA) showed that the antimicrobial activity of the chitosan was directly influenced by an increase in the DDA of the tested chitosan (Chung and Chen, 2008). Furthermore, when Je and Kim (2006) introduced an amino-functionality onto chitosan at the C-6 position by amino-alkylation, an improved antimicrobial activity was observed compared to native chitosan.

Concerning the chitosan polymerization degree, most studies have showen that chitosan with a molecular weight in the 10 – 20 kDa range, have the highest bactericidal activity (Ueno *et al.*, 1997; Liu *et al.*, 2001; Park *et al.*, 2004). In contrast, oligomeric chitosans (pentamer and heptamer) have better antifungal effect than larger units (Kendra and Hadwiger, 1984; Savard *et al.*, 2002). Thereby, it has been shown that the antimicrobial effect of chitosan is more important on fungi than on bacteria. The MICs of chitosan against fungi varried from 0.01 mg/ml for *Botrytis cinerea* and *Fusarium oxysporum* to 0.1 mg/ml for *Drechstera sorokiana*, 1 mg/ml for *Rhizoctonia solani* and 2.5 mg/ml for *Trichophyton equinum* (Liu *et al.*, 2001). In general, chitosan (0.01-2 mg/ml) reduces the *in vitro* growth of numerous fungi with the exception of *Zygomycetes*, which are fungi containing chitosan as a major component of their cell wall (Allan and Hadwiger, 1979).

In addition, some studies showed that chitosan has a stronger bactericidal effect against Gram-positive than against Gram-negative bacteria. For instance, Kumar *et al.*, (2007) determined the minimal inhibitory concentrations (MICs) of chitosan on *Escherichia coli* D21, *Bacillus*

cereus and *Staphylococcus aureus* by turbidity (bacterial growth was visually analyzed) and plating tests (viable counts), and observed that the MIC of chitosan for *E. coli* D21 (Gram-negative) was 3 times higher than that obtained for *Bacillus cereus* and *Staphylococcus aureus* (Gram-positive) (Table 2). This effect was also observed by Gerasimenko *et al.* (2004) who monitored the growth of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* (Gram-negative) and *Lactobacillus plantarum*, *Bifidobacterium bifidum* (Gram-positive) in the presence of different concentrations of chitosan as viable counts (CFU/ml). They determined the MICs of chitosan for these strains as being of more than 10 mg/ml for the Gram-negative bacteria and 0.06 and 0.0005 mg/ml for the Gram-positive bacteria, respectively. Jeon *et al.* (2001) by measuring the turbidity of cultures at 640 nm or by visual and microscopy analysis of the bacterial growth in the presence of chitosan, observed, that the MIC of chitosan for *Streptococcus faecalis* was four times smaller than that for *E. coli* (Table 2). The same tendency concerning the bactericidal effect of chitosan was reported in studies realized by other research groups (No *et al.*, 2002; Chung and Chen, 2008). The MIC values of chitosan depended on the degree of *N*-deacetylation and the molecular weight of chitosan. The MICs of chitosan, obtained by different research teams for various Gram-positive and Gram-negative bacteria when using chitosan with similar degree of *N*-deacetylation and different molecular weight (Mn), are resumed in Table 2.

Table 2. Minimal inhibitory concentration of chitosan against various bacterial strains.

Bacterial strain	MIC mg/ml	DDA %	Mn (kDa)	Reference
Escherichia coli D21	3	85	10	Kumar *et al.*, 2007
Escherichia coli O-157	1.2	90	10	Jeon *et al.*, 2001
Pseudomonas aeruginosa	10	85	12	Gerasimenko *et al.*, 2004
Klebsiella pneumoniae	10	85	12	Gerasimenko *et al.*, 2004
Vibrio parahaemolyticus	4	75	10	Park *et al.*, 2004
Lactobacillus plantarum	0.6	85	12	Gerasimenko *et al.*, 2004
Bifidobacterium bifidum	0.005	85	12	Gerasimenko *et al.*, 2004
Bacillus cereus	1	85	10	Kumar *et al.*, 2007
Staphylococcus aureus	1	85	10	Kumar *et al.*, 2007
Streptococcus faecalis	0.3	90	10	Jeon *et al.*, 2001

The difference in the global electronegativity of the cell wall between Gram-positive and Gram-negative bacteria is assumed to be responsible for this distinctive antibacterial activity. In the cell walls of Gram-positive bacteria, structural acidic polysaccharides such as teichoic and teichuronic acids carry negative charges originating from phosphoryl (teichoic acids) or carboxyl (teichuronic acids) groups. Interestingly, by using a mutated *S. aureus* strain impaired in teichoic acid synthesis, Raafat *et al.* (2008) observed that this mutant was less susceptible to chitosan than the wild type strain. Therefore, in Gram-positive bacteria, one of the direct targets of the chitosan are the negatively charged teichoic acids (Raafat *et al.*, 2008) or teichuronic acids. However, in Gram-negative species, the lipopolysaccharides (LPS) from the outer membrane mask the net charges by providing partial hydrophobicity (lipoprotein A) and by minimizing the exposure of unsubstituted acidic groups. In *E. coli*, the Gram-negative model bacterium, chitosan interacts with negatively charged phospholipids from the cell membrane (Liu *et al.*, 2004). Similarly, in the yeast *Saccharomyces cerevisiae* the NH^{+}_{3} groups of chitosan are thought to electrostatically interact with sphingolipids, the main negatively charged components of the plasma membrane (Zakrzewska et al., 2007).

In the second category of studies, the utilization of sublethal doses of chitosan allowed a better understanding of the mechanism of action of chitosan as stress agent for microorganisms. Two different mechanisms have been proposed to describe the antimicrobial activity. The first mechanism involves the binding of chitosan to DNA which triggers the inhibition of RNA synthesis (Kendra and Hadwiger, 1984). To reach the DNA and to penetrate the cell wall and membrane the chitosan molecule must be smaller than 2-7 nm (Raafat *et al.*, 2008). However, there is no direct evidence to sustain this mechanism.

The second and most accepted mechanism proposes that the positively charged chitosan reacts with the negatively charged microbial cell surface, thereby, altering cell permeability and resulting in nutrients being prevented from entering the cell and/or molecules leaking from the cell (Liu *et al.*, 2004; Chung and Chen, 2008).

Recent studies have shown that the mode of action of chitosan is complex and involves a cascade of events, which eventually leads to bacterial death (Raafat *et al.*, 2008; Zakrzewska *et al.*, 2007). First of all, it was shown by electron microscopy of chitosan-treated bacteria such as *E. coli*, *S. aureus* and *Salmonella typhimurium*, that the bacterial cell surface was thickened, probably by chitosan deposits attached to the negatively charged surface polymers. This interaction was further sustained by the observation that the presence of divalent cations impaired the effect of chitosan, probably by binding to the chitosan targets. Moreover, the cell membrane of *S. aureus* treated with chitosan was locally detached from the cell wall, forming vacuole-like structures most probably due to water and ion efflux (Helander *et al.*, 2001; Liu *et al.*, 2004; Raafat *et al.*, 2008).

Secondly, it was shown that interaction of cationic chitosan with the cell surface alters the integrity of the cellular membrane, which loses its barrier potential. The leakage of small intracellular constituents such as nucleotides and coenzymes (illustrated by the increase of the UV-absorbance at 260 nm of the supernatant fluids), and an increased flux of K^+ are indications of cell membrane permeabilization caused by the chitosan (Liu *et al.*, 2004; Chung and Chen, 2008). This leads to the reduction of the membrane potential, as observed for *S. aureus* exposed to chitosan using DiBAC4 uptake assays. Furthermore, the occurrence of SCVs (small-colony variants) of *Staphylococcus aureus* was observed following exposure to a high concentration of chitosan. SCVs represented a subpopulation of *S. aureus* strains characterized by a reduced membrane electrochemical, gradient which explains their higher resistance to chitosan compared to the parent strain (Raafat *et al.*, 2008). Another interesting study performed by Helander *et al.* (2001) showed that the pH of the chitosan solution had a remarkable influence on the permeabilization of the outer membrane of *E. coli*, *Pseudomonas aeruginosa,* and *Salmonella typhimurium.* Thus, analysis of 1-N-phenylnaphthylamine (NPN) uptake by bacteria exposed to a chitosan solution at pH 5.3 or 7.2 revealed that the chitosan “induced” NPN uptake only at acidic pH. At pH 5.3 chitosan is protonated, whereas the carboxyl and phosphate groups of the bacterial surface are anionic and they become potential

sites for electrostatic binding by chitosan. Furthermore, this pH may also induce an acidic stress for the bacteria.

The outer membrane of Gram-negative bacteria and the cell wall of Gram-positive bacteria are the first barriers that chitosan has to overpass in order to complete its killing action. Permeabilization of the inner membrane, indicated by the release of the cytoplasmic enzyme β-galactosidase, was observed in *E. coli* in relation with variations in chitosan concentration and with the period of exposure to this polymer (Liu *et al.*, 2004). After one hour of incubation in the presence of 5 g/l of *N*-deacetylated chitosan (85% DDA), Liu *et al.* could observe a gradual increase in β-galactosidase release. Interestingly, when Chung and Cheng cultured *E. coli* in the presence of 2.5 g/l of highly *N*-deacetylated chitosan (95% DDA), a release of cytoplasmic nucleotides could be observed only after 8 hours of induction (Chung and Cheng, 2008). Moreover, changes in the intracellular ionic medium also seemed to affect the bacterial nucleoid organization (Raafat *et al.*, 2008). Finally, the interaction between chitosan and bacteria results in bacterial cell death.

In relation with those events, the transcriptional response profiles obtained for microbes grown in the presence of chitosan were analyzed. They indirectly confirmed the observations described above. For instance, thickening of the cell wall and membrane depolarization and permeabilization in *S. aureus* due to chitosan binding caused up-regulation of genes preferentially expressed under oxygen deficiency and interruption of electron transport chain, and of genes involved in nitrate reduction and anaerobic respiration. Moreover, the inhibition of bacterial growth was accompanied by a decrease in the metabolism of carbohydrates, amino acids, lipids and coenzymes, as well as by a decrease in RNA and protein biosynthesis as reflected by the gene expression profiles (Raafat *et al.*, 2008). This transcriptional profile suggests that chitosan, by altering membrane functionality, forced *S. aureus* cells to switch to anaerobic respiration which leads to dysfunction of the whole cellular apparatus and, finally, to death.

The transcriptional response to chitosan was also analyzed for *Saccharomyces cerevisiae*, the model organism for fungi (Zakrzewska *et al.*, 2008). The time-course analysis allowed the identification of over six hundred genes which were up- or down-regulated in response to the chitosan presence. The first genes to be upregulated, after only 15 min of incubation in the presence of chitosan, were genes related to the plasma membrane biogenesis and genes encoding for cell wall anchored proteins. The induction of these genes was accompanied by an increase in the expression of vacuole-formation related genes and in the stress related genes following 30 min and 120 min of incubation, respectively. Most of the downregulated genes were involved in RNA processing and ribosome biogenesis.

All those observations suggest the complexity of the mechanism of action of chitosan on microorganisms. Interestingly, this mechanism is very similar for bacteria and fungi. The chitosan, due to its cationic charge, directly interacts with the negatively charged groups from the bacterial and fungal cell membrane surface. This direct contact triggers other indirect events such as cell wall disorganization, cytoplasmic membrane depolarization and permeabilization accompanied by cellular leakage, without distinct pores formation, and finally cell death.

With time, bacteria adapted to the change in the nutrient disponibility by diversifying their battery of metabolic enzymes. They also adapted to the presence of different growth restrictive molecules such as antibiotics, cationic peptides and metals by changing the constitution of their membrane (Tzeng *et al.*, 2005) and by modifying their genetic information (spontaneous mutations and gene transfer).

General hypothesis of this PhD project: Chitosanases as resistance factors against the antimicrobial effect of chitosan.

Chitosan has a dose-dependent antimicrobial effect. In function of its concentration, this biopolymer can act as a stress agent for bacteria, can inhibit bacterial growth and even cause cell death. The molecular weight is a key factor for the antibacterial activity of chitosan. Chitosan molecules with a molecular weight in the 10 – 20 kDa range have the highest antibacterial activity. On the other hand, the minimal molecular weight required for an antibacterial effect is not well defined, but is thought to be in the 2 - 5 kDa range. Chitosan with small molecular weight (<2 kDa) is devoid of any antibacterial activity; moreover it can support bacterial growth. Chitosanases hydrolyze chitosan with antimicrobial activity (10 – 20 kDa) to small subunits (< 2 kDa) which can be further metabolized by bacteria. In this context, the production of a chitosanase may exert a protective effect against the antibacterial activity of chitosan. Therefore, a chitosanase gene may be considered as a chitosan-resistance gene. The studies concerning the effect of the molecular weight on the antimicrobial activity of chitosan are summarized in the introduction of the first article (Chapter I).

First of all, we analyzed how the expression of an heterologous chitosanase gene, introduced by genetic manipulation, could protect *E. coli* (a bacterium naturally devoid of chitosanase activity) against the antibacterial effect of chitosan (results presented in Chapter I). Next, our interest was focused on the study of organisms possessing a chitosanase gene, bacteria belonging to the *Streptomycetes* group. Taking advantage of a new method for gene disruption developed during my PhD studies (results presented in Chapter II), we could compare the susceptibility of a wild type strain (*Streptomyces lividans* TK24) to that of a mutated strain for chitosanase CsnA production, to the presence of chitosan. Furthermore, we determined the biochemical properties of the chitosanase CsnA (results presented in Chapter III). Finally, we showed a particular cellular localization of the exo-chitosanase CsxA from *Amycolatopsis orientalis*, which could be explained by involvement of the CsxA in the protection against the antimicrobial effect of chitosan (results Chapter IV). This study helped us to better understand

how bacteria adapted to the presence of this antimicrobial compound in their environment and to determine the role played by chitosanases in this adaptation process.

RESULTS

CHAPTER I

1.0 Role of heterologous chitosanase expression in the protection against the antimicrobial effect of chitosan on *Escherichia coli*

Chitosanases are glycoside hydrolases responsible for the cleavage of the β-(1,4)-linkages in partly acetylated chitosan. For more than three decades, bacterial chitosanases have been used to determine the mechanism of chitosan hydrolysis at both biochemical and molecular levels. The chitosanase produced by *Streptomyces* sp. N174 (CsnN174) has been intensively studied in our laboratory. Presently, chitosanases and chitosan are being investigated for their role in the maintenance of the ecological balance in Nature.

Here, we examined the effect of heterologous chitosanase CsnN174 production by *E. coli* on its survival in the presence of chitosan. Chitosan has a large spectrum of antimicrobial activity, as it inhibits the growth of bacteria, fungi and viruses. For qualitative and quantitative results, we used CsnN174 mutants characterized by different relative activities issued from previous mutagenesis studies realized in our laboratory. The bactericidal effect of chitosan depends on its polymerisation and *N*-acetylation degrees as demonstrated in all the studies reviewed in the introduction of this article. In this context, *E. coli* strains expressing heterologous chitosanases characterized by different relative activities were analyzed for their capacity to survive in the presence of chitosan with different degrees of polymerization and *N*-acetylation. Next, we were interested to see how chitosanase secretion may affect the survival of a negative-chitosanase *E. coli* strain in the presence of chitosan.

Overall, this study showed that the chitosanase confered a protection for the producing host toward the bactericidal effect of chitosan and that the degree of this protection was related to chitosanase activity. Moreover, our results showed that expression of the heterologous chitosanase by *E. coli* did not influence the growth of the *E. coli* strain lacking a chitosanase activity, probably due to the fact that, in *E. coli*, this enzyme has an intracellular localization. Thereby, secretion of chitosanase is imperative for the protection of neighbouring bacteria against the antimicrobial activity of chitosan.

These results are presented in the form of an article entitled: *Molecular weight modulates the antimicrobial effect of chitosan on Escherichia coli*. A previous version of this article has been published in EUCHIS 2009. Proceedings of the 9th International Conference of the European Chitin Society, article E1-1.

The contribution of each author is the following: J. Gagnon performed the mutagenesis of the T45 amino acid in CsnN174 as well as the plate streaking test and the spot test with the first set of *E. coli* strains. She also optimized the medium composition and the growing conditions for those experiments. M.-È. Lacombe-Harvey provided the second set of *csnN174* mutants, namely, *csnN174*R42K and *csnN174*D40G+R42K. I optimized the minimal medium and performed the experiments for the determination of the minimal inhibitory concentration (MIC) of chitosan. I also performed all the experiments concerning the role of chitosanase expression for the survival of *E. coli* strains in the presence of chitosan in liquid medium and on solid medium. I determined the relative activities for the second set of mutants in the *E. coli* total cellular extracts. Together with Dr. R. Brzezinski, I analyzed all the results presented in this article and wrote the manuscript. This research work was done under the supervision of Dr. Ryszard Brzezinski.

1.1 Molecular weight modulates the antimicrobial effect of chitosan on *Escherichia coli*

Molecular weight modulates the antimicrobial effect of chitosane on *Escherichia coli*

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ABSTRACT

The antimicrobial activity of chitosan and its derivatives, against bacteria and fungi, has received considerable attention in recent years due to potential applications in food industry, pharmacology and agriculture. The molecular weight of this biopolymer as well as its degree of *N*-acetylation are key parameters of this activity. Lately, due to the special interest that chitosan has received as antimicrobial agent, its mode of action on microorganisms such as bacteria and fungi is better understood. Less is known concerning the role that chitosanases, enzymes catalyzing chitosan depolymerisation, may play on bacterial survival in the presence of chitosan. In this work, we investigated the role of heterologous chitosanase expression on the survival of various *E. coli* strains in the presence of chitosan. We also assessed the effect of chitosanase secretion on the survival of bacteria devoid of chitosanase activity both on solid and liquid media containing chitosan. Our results suggest that chitosanases may play an important role in the resistance of bacteria to the toxic effect of chitosan. This effect is more compelling in the case of secreted chitosanases which exert a protective effect for the producing host as well as for the neighbouring bacteria.

INTRODUCTION

Chitosan – a partly or totally *N*-deacetylated derivative of chitin is present in Nature in the cell walls of several groups of organisms. Fungi belonging to the order *Mucorales* and green algae from the order *Chlorellales* have been the most studied [1-2]. Considering the ubiquity of these organisms, chitosan in Nature can be qualified as being "not abundant but not rare". In the laboratory, many microorganisms are able to use chitosan as sole carbon and/or nitrogen source – as long as they possess the necessary enzymes for the degradation of the long chitosan chains, for the transport of resulting chitosan oligosaccharides and metabolism of their monomeric derivatives. However, due to its low abundance, the significance of chitosan as a nutrient is probably limited to some particular niches. This situation is evolving due to the increasing popularity of chitosan in technology. For instance, tens of tons of chitosan are sold in drugstores in many countries and ingested by humans as pills or capsules. As a consequence, the microflora of the human digestive tract is exposed to relatively high chitosan concentrations and, ultimately, some chitosan forms will be found in septic tanks and sewage water. Most of the microorganisms involved in this new interaction with chitosan have not evolved in contact with this polymer. The use of chitosan in technology can create new selection pressures, influencing the composition of microbial populations.

In this context, the antimicrobial activity of chitosan is particularly relevant. At relatively high chitosan concentrations, differential susceptibility of microorganisms to chitosan may result in a severe rebuilding of the microbial populations. In this work, we examined the effect of the molecular weight of chitosan on its antimicrobial activity and the presence of chitosanase enzymes as a possible factor of resistance of microorganisms to chitosan.

Chitosanase production by a given microorganism will result in the reduction of the degree of polymerization of chitosan. Consequently, the solubility of this polymer as well as its ability to

diffuse in aqueous environments will be increased. Chitosanase activity will thus render chitosan more bioavailable. On the other hand, chitosanase activity could transiently intensify the antimicrobial effect of chitosan unless chitosan is rapidly hydrolyzed into short oligosaccharides. The biological effect of these two contradictory tendencies is difficult to predict.

Ueno *et al.* [3] compared the minimal inhibitory concentrations (MIC) of various chitosan oligomers (degree of acetylation DA= 2%) to *Escherichia coli*. An oligomer of molecular weight (Mw) 10500 was found to be much more toxic (MIC = 40 mg/L) than the shorter (Mw = 5500) form (MIC = 320 mg/L), while the lower Mw oligomers (Mw < 4100) had no effect on *E. coli* growth at 5000 mg/L. Interestingly, oligomers of Mw = 9300, monitored through FITC labelling, were localized to the cell wall while short oligomers penetrated inside the cell and were catabolised. Furthermore, the low Mw fraction stimulated the growth of *E. coli* [3]. Tsai *et al.* [4] compared high molecular weight chitosan (DA = 10%) with an oligosaccharide mixture derived from it by unspecific hydrolysis with cellulase. The mixture was mostly composed of oligomers with a degree of polymerization (DP) of 6 to 8. For a series of foodborne pathogens, the MIC for this oligosaccharide mixture ranged from 5 to 29 mg/L, while the values obtained for unhydrolyzed chitosan were from 50 to 1000 mg/L. For *E. coli*, the MICs were 16 and 100 mg/L respectively. Liu *et al.* [5] prepared, by γ-irradiation, a series of chitosan polymers (DA = 15%) varying in their Mw. Their antibacterial activities have been tested against *E. coli* grown in a liquid medium and monitored by the optical density at 610 nm. Antimicrobial activity increased for chitosans of Mw values ranging between 5,000 and 90,000 but, then, decreased with further increase of the chitosan Mw. In the presence of chitosan of Mw = 50,000, growth was inhibited at a concentration of 100 mg/L or higher. At constant molecular weight, growth inhibition was more severe at lower DA and at acidic pH. A similar tendency in the low Mw range was observed by Jeon *et al.* [6]. In their study, chitosans (DA = 11%) of various Mw were obtained by hydrolysis with chitosanase in an immobilized enzyme reactor followed by membrane ultrafiltration. Three oligosaccharide fractions of Mw <1kDa, 1kDa<Mw<5kDa and 5kDa<Mw<10kDa gave, for *E. coli*, MIC

values of 2500, 1400 and 600 mg/L respectively. Intact chitosan gave a MIC of 250 mg/L in this study. Antibacterial effect decreased with the decrease of molecular weight. The authors concluded that "for effective inhibition, Mw of chitosan should be higher than or around 10,000" [6]. The same tendency was observed by these authors in a more recent work [7].

In summary, with the exception of the study of Tsai *et al.* [4], most studies showed that a decrease in molecular weight in the low range (<10 – 20 kDa) is accompanied by a progressive loss of antibacterial activity. Following these observations, we hypothesized that chitosanase production by a microorganism could result in a protective effect against chitosan, since enzymatic hydrolysis would produce chitosan forms with lesser antimicrobial activity. In this work, we present a study on the influence of chitosanase production on the survival of *E. coli* in the presence of chitosan.

MATERIAL S and METHODS

Bacterial strains and media components.

Escherichia coli strains JM109 and DH5α™ and *Streptomyces lividans* strain 10-164 as well as vectors pALTER-1, pUC19 and cloning methods used for the introduction of wild-type or mutated chitosanase genes in these vectors have been described previously [8, 9]. The chitosanase gene originated from *Streptomyces* sp. N174 (*csnN174*) [10]. Chitosan polymers varying in their molecular weight have been prepared from chitosan obtained from Sigma (DA = 21%) or Diversified Natural Products Canada (DA = 6%) by limited hydrolysis with CsnN174. Chitosan (40 g/L) was dissolved in 0.2 M acetic acid, mixed with chitosanase and incubated at 37°C. After various incubation periods, reaction was stopped by heating in boiling water for 30 min. Number average molecular mass (M_n) was estimated by reducing end assay using the Lever reagent [11]. Chitosan hydrolysates were stored at -20°C without

further purification or sterilization. TY agar medium was used for *E. coli* (per litre): Tryptone 10 g; Yeast extract 5 g; agar 15g. Other components are indicated in the respective tables or figures. Chitosan hydrolyzate was added to hot, freshly autoclaved medium, before pouring on plates.

Determination of minimal inhibitory concentration (MIC) of chitosan.

Minimal agar medium (MAM) ($MgSO_4$ 0.2 g/L, $CaCl_2$ 0.02 g/L, KH_2PO_4 1 g/L, K_2HPO_4 1 g/L, $FeCl_3$ 0.05 g/L and NaCl 2.5 g/L) was supplemented with glucose 1 g/L, $(NH_4)_2SO_4$ 1 g/L, malt extract 1 g/L, ampicillin 100 μg/ml and IPTG 54μg/ml. TY medium was supplemented with 50 mM NaCl, 5 mM $MgSO_4$, 10 mM citrate buffer pH 6, 100 μg/ml ampicillin and 54 μg/ml IPTG. MICs were determined on a series of TY medium or MAM plates containing 0, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9 or 1 g/L chitosan of 10 kDa prepared as described above.

RESULTS

We assessed the effect of chitosanase production by *Escherichia coli* (a bacterium naturally devoid of chitosanase activity) on its survival in the presence of chitosan. In the first series of experiments, *E. coli* strains were transformed with plasmids directing the production of heterologous chitosanases with different relative activities and the behaviour of those strains in the presence of chitosan was analysed. In another series of experiments, we verified the effect of chitosanase secretion on the survival of an *E. coli* strain lacking chitosanase activity.

Plate streaking test.

In these experiments (Table 1), *E. coli* JM109 cells were transformed with the pALTER-1 vector or its derivatives harbouring the following *csnN174* genes: Wild-type (encodes a protein with 100% activity); mutant V148T (10% residual activity) or mutant T45H (<0.1% residual activity). Chitosans varying in Mn and DA values were added to TY medium at a final concentration of 300 mg/L. The *E. coli* strains were streaked on TY-chitosan plates using overnight cultures in liquid TY and growth was estimated after 72 h at 37°C (Table 1). (-) stands for absence of growth, (+) stands for a growth observed in the begining of the streak and (++) stands for a growth observed from the beginig till the end of the streak.

Table 1: Effect of heterologous Csn activity expression on *E. coli* growth on a chitosan containing medium

Strain JM109 / Csn gene	Csn relative activity	Chitosan properties / Growth estimation			
		DA 21% Mn 20 kDa	DA 21% Mn 12 kDa	DA 21% Mn 2 kDa	DA 6% Mn 12 kDa
Vector only	no Csn	-	-	++	-
csn wt	100%	++	++	++	++
csn V148T	10%	+	+-	++	-
csn T45H	<0.1%	-	-	++	-

The results are summaryzed in Table 1. They showed that there is a direct relation between growth of the tested *E. coli* strains on chitosan and the residual activities of the expressed heterologous chitosanases. Furthermore, by analyzing the growth of the mutant V148T we could observe that the 12 kDa chitosan was more toxic than the 20 kDa chitosan. 2 kDa chitosan showed no apparent toxicity and it supported the growth of all the strains tested.

Spot test.

Using a spot test approach, 3 h cultures of *E. coli* strains in liquid TY broth were diluted withresh medium to standardize their optical density. After serial dilution from 10^{-1} to 10^{-5}, 5

μl samples were spotted on chitosan medium. Plates were incubated for 72 h at 37°C. Examples of results are shown in Figure 1. Strains are the same as in Table 1.

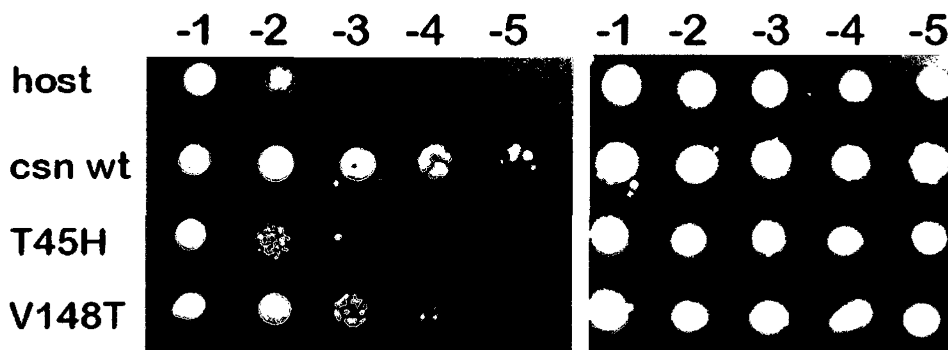

Figure 1: Spot test for growth of *E. coli* strain JM109 expressing various levels of N174 chitosanase. Medium: TY agar with chitosan (500 mg/L; DA = 6%; Mn = 15kDa); 50 mM NaCl and 5 mM $MgSO_4$. Left: medium supplemented with 10 mM citrate buffer pH 6; right: medium with 10 mM phosphate buffer pH 6.

As shown in Figure 1 this test confirmed the results obtained in the plate streaking experiments. The relative activity of the heterologous chitosanases determined the level of resistance of the *E. coli* strains to chitosan. This effect can be observed when the medium composition is well optimized. When phosphate buffer was used, even for the *E. coli* strain devoid of a chitosanase activity we could observe a strong resistance to chitosan (right panel). Phosphate groups are known to form a complex with chitosan. Therefore, NH^{+}_{3} groups in chitosan are not exposed anymore for interaction with the bacterial cell wall, and the toxic effect of chitosan is lost.

Another set of experiments was performed with *E. coli* DH5α™ strains transformed with the pUC19 vector or with its derivatives containing the *csnN174* gene (encodes a protein with 100% activity) or its mutated forms, single mutant *csnN174*R42K (3.1% residual activity) or double mutant *csnN174*D40G+R42K (0.8% residual activity). We determined the MICs of chitosan (Mn ≈ 10 kDa) on TY agar or MAM medium for these strains.

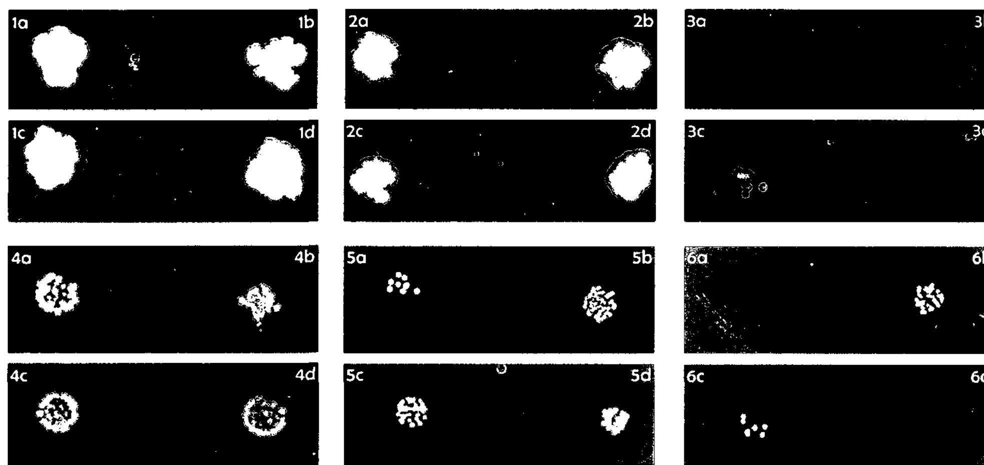

Figure 2: Minimal inhibitory concentration of chitosan for *E. coli* DH5α^{TM} strains expressing chitosanases with different relative activities. TY medium supplemented with 0 g/L (1), 0.3 g/L (2) and 0.5 g/L (3) chitosan. MAM medium supplemented with 0 g/L (4), 0.5 g/L (5) and 0.6 g/L (6) chitosan. 5 µl of the 10^{-5} dilution of each DH5α^{TM} transformant carrying the pUC19 vector (a), pUC19 *csnN174* (b), pUC19 *csnN174*R42K (c) and pUC19 *csnN174*D40G+R42K (d) were spotted on the agar plates and incubated at 37°C for 48h.

As shown on Figure 2, cells harboring a heterologous chitosanase with an activity as low as 3.1% of the maximal value showed a significant protection against the inhibitory effect of chitosan. Similarly to the results shown on Figure 1, for all the tested strains, the MICs of chitosan were dependent of medium composition.

Next, we were interested in determining if expression of chitosanase by a given strain of *E. coli* (pUC19 *csnN174*) could influence survival on a chitosan medium of an *E. coli* strain devoid of chitosanase activity when both strains are grown together. To assess this eventual "altruistic" effect, the tested *E. coli* strains were grown together or separately at 37°C in liquid TY medium supplemented or not with 0.4 g/L chitosan ($M_n \approx 3$ kDa). After various incubation times, growth of both strains was monitored as viable counts (CFU/ml) on LB agar medium with 100 µg/ml ampicillin, 80 µg/ml X-Gal and 54 µg/ml IPTG (Figure 3). Both strains could be distinguished from each other, as the strain expressing the chitosanase formed white colonies, while the strain devoid of chitosanase activity formed blue colonies on this medium.

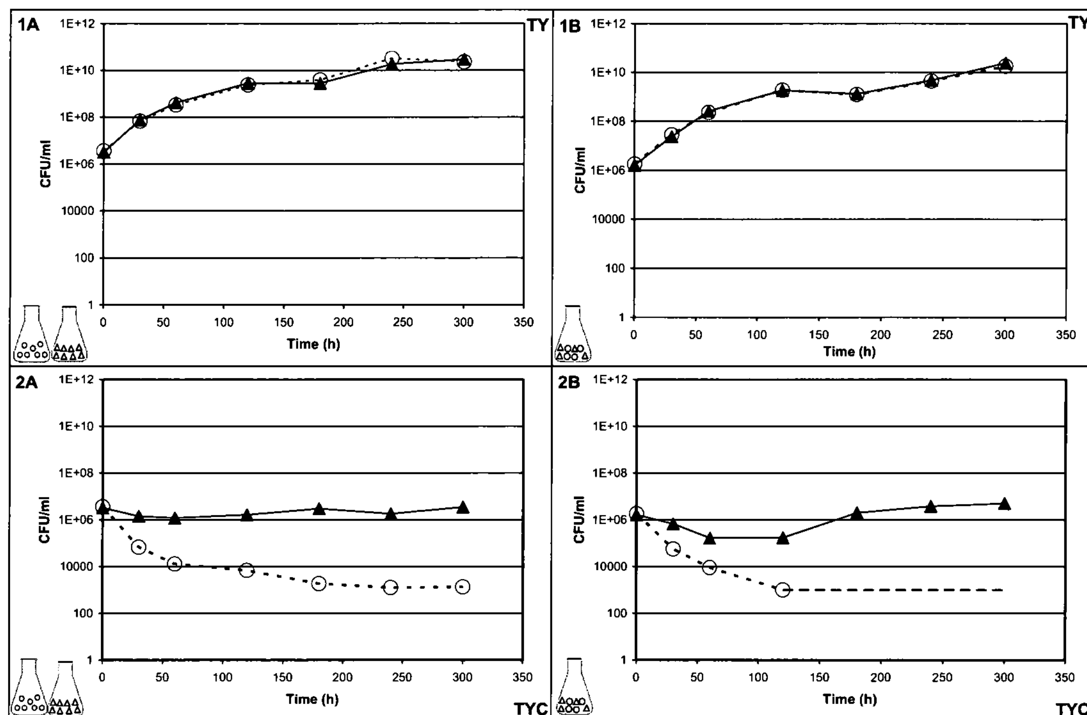

Figure 3: Cell counts for *E. coli* DH5α^{TM} strains expressing (*E. coli* pUC19csnN174) or not (*E. coli* pUC19) a heterologous chitosanase, grown either separately (A) or in co-culture (B) in the absence (TY) or presence (TYC) of chitosan. 1A: Strains grown separately in TY; 1B: strains grown in co-culture in TY; 2A: strains grown separately in TYC; 2B: strains grown in co-culture in TYC.

As shown in the panels 1A and 1B, in the absence of chitosan, the two strains had a very similar growth, when cultured together or either separately. Therefore, it was interesting to see how they would react in the presence of chitosan. As shown in the panels 2A and 2B of Figure 3, the presence of the strain expressing the heterologous chitosanase in the co-culture did not confer any growth advantage to the second strain (*E. coli* pUC19) in the presence of chitosan. This may be explained by the fact that, in *E. coli*, the chitosanase is not secreted into the culture medium, but most probably it remains confined to the periplasmic space of the cell wall. Indeed, after immunoblotting with anti-chitosanase antibodies, we could observe the presence of the chitosanase in crude, whole cell protein extracts of *E. coli* pUC19 *csnN174* but not in the culture supernatants fluids (data not shown).

Finally, we also determined how growth of a chitosanase-negative *E. coli* will be influenced by neighbouring chitosanase-producing bacteria on solid medium. The selected chitosanase producers were: *E. coli* pUC19 *csnN174* (intracellular periplasmic chitosanase) and *Streptomyces lividans* 10-164 pIAF907*csnN174* (extracellular chitosanase). Growth was tested on MAM medium supplemented or not with 0.5 g/L or 0.6 g/L chitosan ($M_n \approx$ 10kDa). For tests with *S. lividans*, spores were streaked on the MAM medium (±chitosan) and plates were incubated for 48 h at 30°C; then the *E. coli* pUC19 (no chitosanase) strain was streaked in the proximity of the actinomycete and the plates were further incubated for 48 hours at 30°C. For tests with *E. coli* pUC19 *csnN174,* this chitosanase-positive strain was allowed to grow for 6 h on the Petri plate before addition of the chitosanase-negative *E. coli* pUC19 strain.

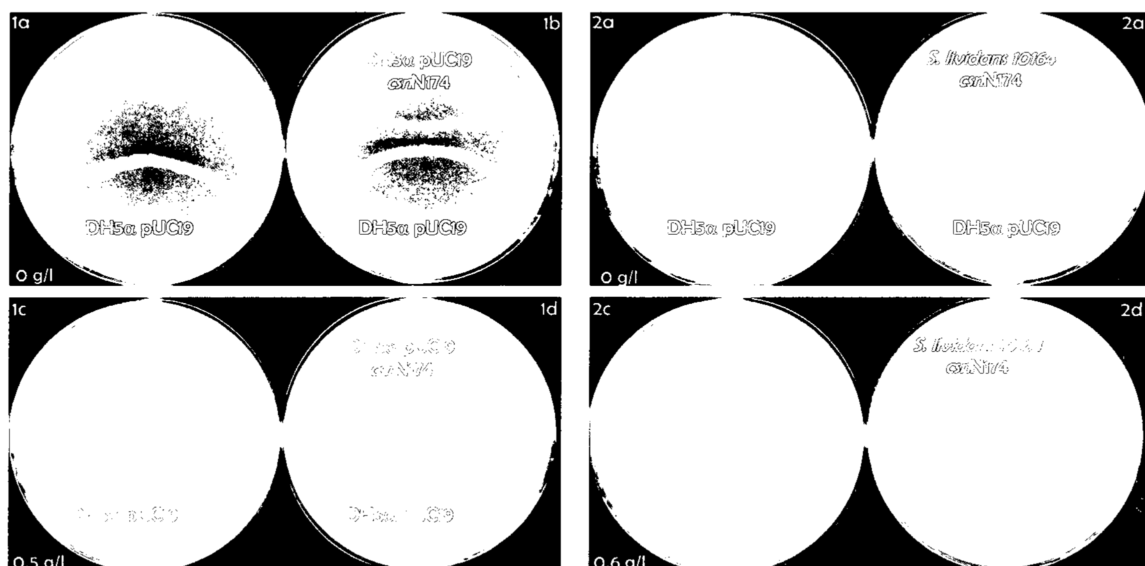

Figure 4: Influence of heterologous chitosanases expression by *E. coli* or *S. lividans* strains on the survival of chitosanase-negative the *E. coli* strain in the presence of chitosan. Influence of *E. coli* pUC19 *csnN174* (1) and *S. lividans* 10164 pIAF907*csnN174* (2) strains expressing heterologous chitosanases on the survival of *E. coli* pUC19 grown in their neighbourhood in the absence (1a-1b; 2a-2b) or presence (1c-1d; 2c-2d) of chitosan. Chitosan concentrations: 0.5 g/L (1c-1d); 0.6 g/L (2c-2d).

As shown on Figure 4, *Streptomyces lividans* 10-164, secreting the heterologous chitosanase in the culture medium, conferred a protection against chitosan for the *E. coli* pUC19 strain

even at a concentration of 0.6 g/L of chitosan. Such a protective effect was not really observed with the *E. coli* pUC19 *csnN174* strain, producing a periplasmic chitosanase.

We conclude that:

Chitosanase expression is an important factor determining the level of resistance of *E. coli* to chitosan. These observations suggest that the protection conferred by chitosanases expression in *E. coli*, against the toxic effect of chitosan, may be exploited in different fundamental studies. For instance, in a study, based on saturation mutagenesis, of the accessory catalytic residue T45 in CsnN174 from *Streptomyces* sp. N174, the utilization of the chitosan medium allowed a fast selection of a single active mutant in which the residue T45 was replaced by a serine [12].

Secretion of an extracellular chitosanase can protect neighboring bacteria against the antimicrobial effect of chitosan by hydrolyzing the polymer into oligosaccharides devoid of inhibitory properties. This protective effect is not observed when the chitosanase remains confined inside the cells.

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CHAPTER II

2.0 Development of a new system for gene disruption and replacement in *Streptomyces* and other actinobacteria with cytosine deaminase as a negative selection marker.

The studies presented in the first chapter suggested that chitosanases could play a role in the protection of the producing bacterial strains against the antimicrobial activity of chitosan. Next, we decided to study more in details these newly discovered characteristics of chitosanases by a genetic study of a naturally chitosanase-positive actinomycete, *Streptomyces lividans* TK24. To achieve this, it was necessary to obtain a chitosanase-negative mutant strain by gene disruption of the chitosanase *csnA* gene from *Streptomyces lividans* TK24.

Suicide selection markers are valuable tools facilitating gene targeting and replacement procedures based on double homologous recombination. To date, few systems based on various negative selection markers have been proposed for genetic manipulation in streptomycetes. Unfortunately, all those systems require the introduction of specific mutations prior to genetic manipulation experiments. Moreover, within the bank of cosmids available for *csnA*, this gene was found at one of the cosmid's extremities. This location is not favourable for further genetic manipulations since a certain length of homologous sequence is needed to permit the homologous recombination to take place on both sides of the gene of interest.

Therefore, the objective of this study was to develop a new system for gene disruption and replacement in *Streptomyces*. These genetic modifications are the result of two homologous recombination events. The new system is based on a vector which contains the aminoglycoside resistance gene for positive selection of the first homologous recombination

event. Early observations from our laboratory showed that *Streptomyces lividans* strain TK24 is resistant to 5-fluorocytosine while being sensitive to 5-fluorouracil when cultivated on ususal agar media, suggesting that cytosine deaminase activity could be absent from this strain. Therefore, cytosine deaminase was chosen as negative selection marker. This enzyme converts the 5-flurocytosine (5-FC), a non toxic prodrug, into 5-fluorouracil, a very toxic antimetabolite which interferes with the normal division and functions of cells. For that, a synthetic gene (*codAS*), encoding the CodA (cytosine deaminase) protein from *Escherichia coli*, optimized for expression in *Streptomyces* was included in the vector.

This new system was developed in order to facilitate genetic manipulations in *Streptomyces*. Gene disruption of the chitosanase gene (*csnA*) was successfully achieved in *S. lividans* TK24 with this new counterselection system.

The results are presented in the form of an article entitled: Cytosine deaminase as a negative selection marker for gene disruption and replacement in *Streptomyces* and other actinobacteria. A short version of this article has been published in Applied and Environmental Microbiology.

Several authors contributed to the realization of this work. The contributions of each author are described above. M.P. Dubeau realized the construction of the vectors pMP101, pMP201, pMP301 and pMP302. She also performed all the biomolecular manipulations required for gene disruption and replacement of the 2657h gene in *S. lividans*. P.E. Jacques performed bioinformatic analysis of several genome sequences from actinobacteria in order to find rare restriction sites, further used for the construction of two polylinkers introduced in the pMP201 vector. N. Clermont participated in the optimization of the conjugation protocol. I optimized and performed the experiments for the determination of the minimal inhibitory concentrations (MICs) of 5-FC, 5-FU and kanamycin for wild type actinomycete strains and for the

Streptomyces lividans TK24 derivatives constructed in this study. I also realized the directed mutagenesis of residue D314 of the *codAS* gene, which became *codASM.* Furthermore, I replaced the *codAS* gene by the *codASM* gene in the vectors described above, thereby, generating the vectors pMG201M, pMG301M, pMG302M and pMGi-*codASM* (integrative vector). I also realized all the biomolecular manipulations required for gene disruption of the chitosanase gene from *S. lividans* TK24. Together with Dr. R. Brzezinski and MP Dubeau, I participated in the preparation of the following manuscript.

Dubeau, M.P.*, Ghinet, M.G.*, Jacques, P.E., Clermont, N., Beaulieu C., Brzezinski R. (2009). Cytosine deaminase as a negative selection marker for gene disruption and replacement in Streptomyces and other actinobacteria. App. Environ. Microbiol. 75 (4):1211-1214. (*These authors contributed equally to this work)

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2.1 Cytosine deaminase as a negative selection marker for gene disruption and replacement in *Streptomyces* and other actinobacteria

Cytosine deaminase as a negative selection marker for gene disruption and replacement in *Streptomyces* and other actinobacteria

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ABSTRACT

One of the current limitations in genetic manipulations of streptomycetes and other actinobacteria is the lack of negative selection markers that can be used without the introduction of specific mutations in the host strain. We here report the development and application of a counterselection system based on cytosine deaminase (CodA). This enzyme can transform 5-flurocytosine (5-FC) into 5-fluorouracil (5-FU), a compound highly toxic for most living cells. Among sixteen actinobacterial species tested, all were resistant to 5-FC, most being sensitive to 5-FU suggesting, that they are naturally devoid of cytosine deaminase. We propose a series of novel vectors for actinobacteria that include a synthetic gene encoding the CodA protein from *Escherichia coli* optimized for expression in *Streptomyces*. When CodA-positive cells are exposed to 5-FC, they are subject to potent negative selection pressure. Gene disruptions and the introduction of an unmarked in-frame deletion were successfully achieved with these vectors. The D314A-mutated version of this gene (*codASM)* is particularly useful as it requires only low levels of 5-FC (20 - 50 μg ml^{-1}) for counterselection. This is the first application of the cytosine deaminase suicide selection system in bacteria.

INTRODUCTION

Cytosine deaminase (EC 3.5.4.1) is an enzyme widely distributed in bacteria and fungi. It catalyzes the hydrolytic deamination of cytosine to uracil as part of the pyrimidine salvage pathway for the recycling of RNA degradation products (40). The absence of cytosine deaminase in mammalian cells combined to the ability of this enzyme to convert 5-fluorocytosine into 5-fluorouracil, a highly toxic compound, led to the development of gene-directed enzyme/prodrug strategies for cancer therapy (6, 14). With such approaches, a bacterial cytosine deaminase gene is delivered to tumour cells and the expressed enzyme converts the 5-fluorocytosine prodrug into a potent cytotoxic molecule.

The suicide effect of cytosine deaminase activity may also be exploited in negative selection procedures for the genetic manipulation of various organisms. Cloning vectors based on this principle have been constructed for mammalian cells (38), plants (45), *Saccharomyces cerevisiae* (19) and *Plasmodium falciparum* (3). Several of these vectors included modified versions of the cytosine deaminase gene (*codA*) from *Escherichia coli* (7). However, to our best knowledge, this negative selection principle has not been proposed so far for bacterial organisms.

Suicide selection markers are valuable tools facilitating gene targeting and replacement procedures based on double homologous recombination. While many such markers are available for a variety of model organisms, the choice is very limited for streptomycetes and other actinobacteria. An attempt to adapt for *Streptomyces lividans* the widely used sucrose sensitivity effect based on the expression of the levansucrase gene (*sacB)* from *Bacillus subtilis* (13) was unsuccessful (27). The glucose kinase gene (*glkA*) of *Streptomyces coelicolor* A3(2) can be counterselected by resistance to 2-deoxyglucose (12) in strains lacking glucose kinase activity. To be efficient, this negative selection must be performed in strains from

which the genomic segment encoding GlkA has been deleted (12, 28). Another negative selection procedure proposed for actinomycetes was based on the observation that a wild type *rpsL* gene (encoding the ribosomal protein S12) confers dominant streptomycin-sensitive phenotype in a streptomycin resistance background; the latter being often obtained by spontaneous mutations of the *rpsL* gene (32, 41). The usefulness of this method has been demonstrated for *Streptomyces roseosporus* (24) but its further use in actinomycetes remained limited to this sole species (5). As an extension of this strategy, it was recently observed that several spontaneous mutants resistant to spectinomycin in *S. roseosporus* occurred in the *rpsE* gene encoding another ribosomal protein, S5. This opens the way for developing a negative selection system based on a spectinomycin-sensitive dominant phenotype (20).

All these negative selection procedures require the use of specifically mutated host strains. Even if the disrupted/replaced genes may be moved into a wild-type genetic background by conjugation (28), this constitutes a serious limitation for many studies. Thus, so far, most gene disruption/replacement procedures based on two subsequent homologous recombination events include a final colony screening step to obtain isolates that have lost an antibiotic-resistance marker. Hundreds to thousands of colonies may be involved in this laborious step, depending on the frequency of the final recombination event. This step persists even with the more recent procedures based on PCR targeting (16, 17). As with other microorganisms, a counterselection-based procedure would greatly facilitate obtaining the desired genomic constructs.

This work is based on an early observation in our laboratory (R. Brzezinski, unpublished) that *Streptomyces coelicolor* A3(2) strain M145 and *Streptomyces lividans* strain TK24 are resistant to 5-fluorocytosine while being sensitive to 5-fluorouracil when cultivated on popular agar media, suggesting that cytosine deaminase activity could be absent from these strains in the tested culture conditions. This was sustained by the fact that there is no apparent *codA* ortholog (gene in different species that evolved from a common ancestral gene by speciation,

and retained the same function in the course of evolution) in the genome sequence of *S. coelicolor* A3(2) M145 (1). Recently, Hughes *et al.* (25) performed a detailed study on pyrimidine salvage pathways in two streptomycete species and noted the absence of cytosine deaminase. Therefore, it would be possible to use the cytosine deaminase-based counterselection procedure in streptomycetes without any genetic modification of the host strain. We report herein a new version of the *codA* gene optimized for expression in actinobacteria and its use in newly constructed vectors. Transformation or conjugation-based vectors are described. We also demonstrate the usefulness of the mutated (D314A) cytosine deaminase gene (34) for counterselection.

MATERIALS AND METHODS

Bacterial strains and plasmids, media and culture conditions.

Bacterial strains and plasmids used in this work are detailed in Table 1. *E. coli* strain DH5α was used for cloning experiments and DNA propagation. The methylase-negative mutant *E. coli* ET12567 containing the non-transmissible pUZ8002 plasmid (33) was used as donor in conjugation experiments. *E. coli* strains were grown on Luria-Bertani broth (LB) supplemented, when necessary, with 100 μg ampicillin (Ap) ml^{-1}, 500 μg hygromycin (Hm) ml^{-1}, 50 μg kanamycin (Kan) ml^{-1} or 25 μg chloramphenicol (Cm) ml^{-1}. Standard methods were used for *E. coli* transformation, plasmid isolation and DNA manipulation (42). Preparation of *S. lividans* TK24 protoplasts and transformation using rapid small-scale procedure and R5 regeneration medium was performed as described previously (28). Transformants carrying the *hyg* or *neoA* resistance gene were selected after addition of 5 mg Hm or Kan to 3 ml of soft agar overlay. Transformants were chosen following two subsequent cycles of purification on solid yeast/malt extract medium (28) with 250 μg Hm or Kan ml^{-1}. Conjugation was done with spores of *S. lividans* TK24 following a published protocol (28). 5 x 10^7 spores were heat shocked in 500 μl 2x YT media then recovered by centrifugation and transferred on 0.22 μm cellulose filter on SLM3 agar medium (9) with 10 mM $MgCl_2$ at 30°C

for 30 min. *E. coli* cells were recovered from an early log-phase culture (OD_{600} ~0.4-0.6) by centrifugation, washed 2 times in 2xYT medium and transferred on the same filter. Usually, donor: receiver ratios between 1:1 and 40:1 were tested. The filters were incubated for 5h at 30°C. Post-conjugative cells were recovered in 1 ml of 2xYT medium and 100 μl samples of cell suspension were spread on SLM3 agar plates and incubated for 12-18h at 30°C. The plates were then overlaid with 2.5 ml of soft agar containing 5 mg Kan and 0.5 mg nalidixic acid per plate and further incubated at 30°C until colony apparition. Exconjugants were purified on solid yeast/malt extract medium with 100 μg Kan ml^{-1} and 25 μg nalidixic acid ml^{-1}. Sporulation was obtained by heavy inoculation of plates with mannitol soya flour medium (28) or SLM3 agar medium (9). Spores were collected with glass beads and stored in 20% glycerol at -20°C.

Table 1: Bacterial strains and plasmids used in this work.

Strain or plasmid	Genotype / description	Reference / source
E. coli DH5α™	$F^-\phi$80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-,m_k^-) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Invitrogen
E. coli ET12567 (pUZ8002)	*dam13* ::Tn*9* *dcm6* *hsdM* *hsdR* *recF143* *zjj201* ::Tn*10* *galK2* *galT22* *ara14* *lacY1* *xyl5* *leuB6* *thi1* *tonA31* *rpsL136* *hisG4* *tsx78* *mtli* *glnV44* F^-	28, 33
Streptomyces lividans TK24	*str-6* $SLP2^-$ $SLP3^-$	28
Streptomyces coelicolor A3(2) M145	$SCP1^-$, $SCP2^-$	2
Streptomyces avidinii	Wild type	ATCC 27419
Streptomyces clavuligerus	Wild type	NRRL 3585
Streptomyces antibioticus Tü 1718 (SKB)	Wild type	47
Nocardia lactamdurans	Wild type	NRRL 3801
Streptomyces fradiae	Wild type	ATCC 31669
Amycolatopsis orientalis subsp. *orientalis*	Wild type	ATCC 19795
Streptomyces avermitilis	Wild type	ATCC 31267
Streptomyces venezuelae	Wild type	ATCC 15439
Streptomyces scabiei EF-35	Wild type	11

Streptomyces melanosporofaciens EF-76	Wild type	11
Nocardioides sp. N106	Wild type	35
Mycobacterium smegmatis MC^2 155	Wild type	44
Saccharopolyspora erythraea	Wild type	ATCC 11635
Streptomyces griseus B-2682	Wild type	NRRL B-2682
pHM8a	Integrative vector	37
pFDNeo-S	*E. coli* plasmid; aminoglycoside resistance gene cassette from Tn5 with a synthetic promoter	8
pMP-codAS	Derivative of pFDNeo-S in which the Neo-S cassette has been replaced by *codAS*	This work
pMP-neoA	Derivative of pFDNeo-S in which the synthetic promoter has been replaced by the *aacC4* gene promoter	2, this work
pMPi-codAS	Integrative vector pHM8a with *codAS* insert	This work
pMGi-codASM	Integrative vector pHM8a with *codASM* insert	This work
pMPi-neoA	Integrative vector pHM8a with *neoA* insert	This work
pMP101	First generation vector; *codAS neoA*	This work
pMP201	Second generation vector, *codAS neoA*	This work
pMG201M	Second generation vector, *codASM neoA*	This work
pMP301	Conjugative vector, *codAS neoA oriT*	This work
pMP302	Conjugative vector, *codAS neoA bla oriT*	This work

Determination of minimal inhibitory concentration (MIC) of 5-fluorocytosine and 5-fluorouracil.

5-fluorocytosine (5-FC) was prepared as stock solution of 15 mg ml^{-1} in distilled water and sterilized by filtration. 5-fluorouracil (5-FU) was prepared as stock solution of 5 mg ml^{-1} in

DMSO. MICs were determined by the agar dilution method (4) on minimal agar medium plates (23) supplemented with 1 g l^{-1} casamino acids, 20 g l^{-1} glucose and 1.2 μM riboflavin and 20 – 800 μg ml^{-1} of 5-FC or 0.2 – 40 μg ml^{-1} of 5-FU. Suspensions of spores (for streptomycete strains) or vegetative cells (*Amycolatopsis sp.* or *Mycobacterium sp.*) were diluted to $2x10^3$ cfu ml^{-1}, spotted on the agar plates containing 5-FC or 5-FU and incubated at 30°C for 72 h.

Vector construction.

The sequence of the cytosine deaminase gene optimized for expression in actinomycetes (*codAS)* has been conceived by reverse translation of the CodA protein sequence from *E. coli* (7) followed by codon optimization for *S. lividans* using JCat (15). A *Sph*I site was created at the initiation codon (CGATGC) resulting in an S to P mutation of the second residue of translated sequence. Another *Sph*I site was introduced immediately after the stop codon. The coding segment was synthesized by Geneart AG (Regensburg, Germany). For transcription initiation, the synthetic promoter from plasmid pFDNeo-S (8) was amplified by PCR with primers 5'-GGCAACTAGCTCGAGGTTGACAT-3' and 5'-TTGTTCAAGCATGCGAAACGATCC-3' (the latter introduced a *Sph*I site at the start codon). The amplified fragment was digested with *Xho*I and *Sph*I and ligated with the *Sph*I-*Sph*I synthetic construct and *Xho*I-*Sph*I-digested plasmid pFDNeo-S, giving pMP-codAS. A mutated version of the synthetic gene (*codASM*), harbouring the D314A mutation (34) was created by PCR-directed mutagenesis (22) by replacement of the GAC codon by the GCC codon, resulting in pMG-codASM.

To change the promoter of the aminoglycoside resistance gene (*neo*) in plasmid pFDNeo-S, two oligonucleotides (5'-TCGAGTATTTGCAACAGTGCCGTTGATCGTGC-3' and 5'-GATCTCAGTCGATCATAGCACGATCAACGGCA-3') were annealed and ligated to the *Xho*I-*Bgl*II-digested plasmid pFDNeo-S. As a result, a segment identical to the *aacC4* gene

promoter (2) was inserted upstream of *neo* coding sequence. This newly configured gene was named *neoA* and the resulting plasmid pMP-NeoA.

To verify resistance levels in strains having only one copy of *codAS*, *codASM* or *neoA* inserted in their genome, we first modified the integrative vector pHM8a (37) to introduce new restriction sites. pHM8a was deleted for its *Bam*HI restriction site by digestion, treatment with the Klenow fragment of DNA polymerase I from *E. coli* and self ligation. Then, the multiple cloning site from pUC19 (New England Biolabs) was sub-cloned as an *Eco*RI - *Hin*dIII fragment between the *Sph*I and *Hin*dIII sites of the pHM8a *Bam*HI-deleted derivative, resulting in the pHM8aBΔM plasmid. Finally, the *codAS*, *codASM* or *neoA* genes were excised by digestion with *Bam*HI and *Hin*dIII from pMP-codAS, pMG-codASM and pMP-NeoA respectively and ligated with the pHM8aBΔM plasmid digested with *Bam*HI and *Hin*dIII, giving pMPi-codAS, pMGi-codASM and pMPi-neoA.

Construction of vectors pMP101, pMP201, pMG201M, pMP301 and pMP302.

The *codAS* cassette was excised with *Hin*dIII and *Eco*RI from pMP-codAS and introduced between *Hin*dIII and *Mfe*I sites of the Litmus 38i plasmid (10), generating pMP100. The *neoA* cassette was added as a *Hin*dIII – *Eco*RI segment (from pMP-neoA) by ligation to pMP100 restricted with *Mlu*I and *Eco*RI resulting in plasmid pMP101amp (Ap^R Kan^R *codAS*). The Ap^R resistance gene and the origin of replication of phage M13 (remaining from Litmus 38i) were deleted from pMP101amp by *Bsp*HI digestion followed by self-ligation, generating the pMP101 vector (Kan^R *codAS*).

To obtain pMP201, two partly complementary oligonucleotides (5'-AGC TTC CTG CAG GAG TCA CTA GTC GAT CTT AAG ATG CAT TGA CG-3' and 5'-AAT TCG TCA ATG CAT CTT AAG ATC GAC TAG TGA CTC CTG CAG GA-3') were annealed and ligated to

the *Hind*III–*Eco*RI-digested pMP101. A second pair of oligonucleotides (5'-CTA GAT CAG TCC GGA GCT ATG TAC AAC TGC AAT TGC TGA GCT AGC GCA TCC TAG GAG TCC GAT-3' and 5'-CGG ACT CCT AGG ATG CGC TAG CTC AGC AAT TGC AGT TGT ACA TAG CTC CGG ACT GAT-3') were annealed and ligated to this first derivative digested with *XbaI* and *Pvu*I, generating pMP201. Then, pMG201M harbouring the *codASM* gene was constructed ligating an *Aat*II-*Hin*dIII *codASM* cassette into the pMP201 vector cleaved with the same enzymes.

To obtain a conjugative vector, the transfer origin *oriT* was PCR-amplified from pIJ773 vector DNA (16) using primers 5'-GTTAACTTGGTGTATCCAACGGCGTCA-3' and 5'-TCATGATTCGAAGTTCCCGCCAGC-3', then, cloned into the pCR2.1 vector using the TA-cloning procedure (Invitrogen). pCR2.1-*oriT* was digested with *Bsp*HI and *Hpa*I and the purified *oriT* fragment was then ligated with the pMP201 *Bsp*HI-*Hpa*I-digested vector, giving pMP301 (Kan^R *codAS oriT*). To allow the use of the *codAS*-based system with *E. coli* donor strains that are resistant to kanamycin, an Ap^R derivative has been constructed. The *bla* gene (Ap^R) was PCR-amplified from Litmus 38i using primers 5'-GCGGGCTTTTTTTGTTAACTACGTC-3' and 5'-AACCGGGGTAAGTTAACCTAAAGTAT-3'. The amplified *bla* fragment and pMP301 were digested with *Hpa*I and ligated, giving pMP302 (Ap^R Kan^R *codAS oriT*).

Gene disruption and replacement.

All DNA fragments for homologous recombination were obtained by PCR amplification using *S. lividans* TK24 genomic DNA. Their identity was confirmed by sequencing.

Construction of pMP101-Ω2657h.

This plasmid was used for gene disruption of the homolog of SCO2657 gene in *S. lividans*

TK24. The first fragment was amplified using primers 5'-TCACCAAGCTTATAGAGCGGATGA-3' and 5'-GGCCGGGTTGAATTCGTAGAGCTG-3' and introduced between *Hin*dIII and *Eco*RI sites of pMP101. The second fragment was amplified using primers 5'-CTGCTCCGGACCTACGCCA-3' and 5'-TGTTGCGGTAGGCCTCCAGCTTCT-3' and ligated between *Bsp*EI and *Stu*I of pMP101 with the first fragment inserted, giving pMP101-Ω2657h.

Construction of pMP302-F2657h.

This plasmid was used for the introduction of an unmarked, in-frame deletion in the SCO2657-homolog in *S. lividans* TK24. The first fragment was amplified with primers 5'-TCCGGAGCTGATAGAGCGGATGAACC-3' and 5'-GCTAGCTTGACCTCGTAGAGCTGGG-3' and ligated between the *Bsp*EI and *Nhe*I sites of pMP302. The second fragment was amplified using primers 5'-GCTAGCGGTGGTCCTGAGCG-3' 5' and 5'-CCTAGGCACTGGGACATGTAGTGGT-3' and ligated into *Nhe*I and *Avr*II of pMP302 with the first fragment inserted, generating pMP302-F2657h. Both amplified fragments were first cloned into the pCR2.1 vector before sub-cloning into pMP302.

Construction of pMG201M-ΩcsnA plasmid.

This plasmid was used for gene disruption of the homolog of SCO0677 (*csnA*) gene in *S. lividans* TK24. The first fragment was amplified with primers: 5'-AAGCTTCTGCCTCTACGCGATCTA-3' and 5'-GCCGGAACAGAATCCGATGAT-3' and ligated into *Hin*dIII and *Eco*RI sites of pMG201. The second fragment was amplified using the primers 5'-TCCGGAGCGGGACCGCGTGTACTT-3' and 5'-GACGCTCAAGGCCTCCCACGA-3' and ligated into *Bsp*EI and *Stu*I sites of pMG201.

Mutant creation.

Plasmids pMP101-Ω2657h and pMG201M-Ω*csnA* were introduced into *S. lividans* TK24 by protoplast transformation while conjugation was used for pMP302-F2657h. After selection of single crossover Kan^R colonies, the presence of *neoA* or *codASM* was verified by PCR amplification on genomic DNA isolated from selected colonies using, respectively, primers sets 5'-TGCCCTGAATGAACTACAGGACGA-3' and 5'-TATCACGGGTAGCCAACGCTATGT-3' for *neoA* and 5'-CAGACCCTGAAGTGGCAGAT-3' and 5'-CATGCTCAGCGCTTGTAGTC-3' for *codAS* or *codASM.* All double crossovers were selected by plating up to 500 spores/plate of Kan^R clones on minimal agar medium supplemented with 5-FC (200 μg ml^{-1}) and Kan (250 μg ml^{-1}) for *2657h* and *csnA* gene disruptions or 5-FC only for *2657h* in-frame deletion. All mutants were verified by PCR and Southern blotting. Southern blotting was done according to the protocol supplied by the manufacturer (Roche Applied Sciences, Switzerland). Probes for hybridization were prepared using DIG-High Prime (Roche Applied Sciences, Switzerland) after two PCR amplifications. Anti-digoxigenin-AP conjugate and NBT/BCIP Stock Solution from Roche Applied Sciences were used for detection.

DNA sequence analysis.

DNA sequencing was performed at the McGill University (Genome Quebec Innovation Center, Canada). The sequence of *codAS* has been deposited in the NCBI database under accession number EU099038.

RESULTS AND DISCUSSION

Resistance to 5-fluorocytosine is frequent among actinobacteria.

Growth on minimal media of cytosine deaminase-positive bacterial species such as *Escherichia coli* or *Salmonella typhimurium* is inhibited in the presence of 10 – 20 μg ml^{-1} of

5-FC (39). In contrast, concentrations as high as 400 – 800 μg ml^{-1} were necessary to inhibit growth of the actinobacteria tested in this work (Table 2). Several strains grew even at the highest tested concentration of 800 μg ml^{-1}. Most strains, however, remained very sensitive to the toxic effect of 5-FU. The absence of cytosine deaminase observed in *S. coelicolor* and *S. griseus* (25) seems to be frequent, perhaps generalized among actinobacteria. The CodA-based counterselection system could then be applicable to a vast array of species.

Table 2: Minimal inhibitory concentrations (MICs) of 5-fluorocytosine and 5-fluorouracil determined for wild type strains.

Strain	MIC 5-FC (μg ml^{-1})	MIC 5-FU (μg ml^{-1})
Streptomyces lividans TK24	≤ 800	< 0.1
Streptomyces coelicolor A3(2) M145	≤ 800	< 0.1
Streptomyces avidinii ATCC 27419	> 800	≤ 40
Streptomyces clavuligerus NRRL 3585	≤ 800	< 0.1
Streptomyces antibioticus Tü 1718 (SKB)	> 800	≤ 40
Nocardia lactamdurans NRRL 3802	≤ 800	≤ 10
Streptomyces fradiae	≤ 400	< 0.1
Amycolatopsis orientalis subs. orientalis ATCC 19795	≤ 800	≤ 5
Streptomyces avermitilis ATCC 31267	> 800	> 40
Streptomyces scabiei EF-35	> 800	≤ 0.8
Streptomyces melanosporofaciens EF-76	>800	< 0.1
Nocardioides sp. strain N106	> 800	≤ 0.4
Mycobacterium smegmatis MC^2155	> 800	20
Saccharopolyspora erythraea ATCC 11635	≤ 400	< 0.1
Streptomyces venezuelae ATCC 15439	≤ 800	< 0.1
Streptomyces griseus B2682	≤ 800	< 0.1

The concentrations tested for MICs determination were: 20, 30, 40, 50, 100, 200, 400, 800 μg/ml for 5-FC and 0.1, 0.2, 0.4, 0.8, 1.6, 2.5, 5, 10, 20, 40 μg/ml for 5-FU.

Cytosine deaminase from *E. coli* increases 5-FC sensitivity in *S. lividans*.

The *codA* gene from *E. coli* had to be rebuilt before introduction into *S. lividans* for two reasons. First, the *codA* gene in *E. coli* has no promoter for initiation of transcription localized immediately upstream of the coding sequence, as its coding sequence is preceeded by the (partly overlapping) coding sequence of the *codB* gene, forming a short two-member operon, *codBA* (7). Second, the original *codA* sequence contains 53.4% G+C, including eight Leu-TTA codons (7), which could interfere with its efficient translation in G+C-rich organisms (30). The codon adaptation index (43) of the original sequence, calculated using the codon usage table of *S. lividans*, was only 0.271. We thus decided to synthesize *de novo* the entire coding sequence for the CodA protein using the preferred codon usage of *S. lividans*, reaching a codon adaptation index of 0.991. The synthetic DNA segment terminated with a *SphI* restriction site at both ends. This resulted in a change from Ser to Pro for the second residue of CodA. The widely tested synthetic promoter segment from the pFDNeo-S vector (8), active in both *E. coli* and actinomycetes was then introduced upstream of the coding sequence, giving the *codAS* gene.

As *codAS* was created essentially for use in procedures including homologous recombination, we determined the phenotype resulting from the introduction of a single copy of the gene per cell. The *codAS* was subcloned into the modified integrative vector pHM8a (37) and the resulting plasmid pMPi-codAS was introduced by transformation followed by site-specific recombination into the IS117 integration site of *S. lividans* (21). *S. lividans* TK24[pMPi-codAS] had a growth and sporulation phenotype indistinguishable from the parent strain. However, its MIC for 5-FC was lowered to 200 μg ml^{-1} (Table 3). The MIC for 5-FU remained lower than 0.1 μg ml^{-1}.

Table 3: Minimal inhibitory concentrations (MICs) determined for *Streptomyces lividans* TK24 derivatives constructed in this study.

TK24 derivative	MIC (5-FU)	MIC (5-FC)	MIC (Kan)
TK24[pMPi-neoA]	≤ 0.1	800	600
TK24[pMPi-codAS]	≤ 0.1	200	≤6.25
TK24[pMGi-codASM]	≤ 0.1	20	≤6.25
TK24 Δ*2657h::neoA* (clones 2 & 9)	≤ 0.1	800	600
TK24 Δ*csnA::neoA*	≤ 0.1	800	600
TK24 Δ*2657h*	≤ 0.1	800	≤6.25

Concentrations tested for MICs determination were the same as in Table 2 for 5-FC and 5-FU. MICs for Kan were tested at 6.25, 12.5, 25, 50, 100, 200 and 300μg ml^{-1}.

Use of the *codAS* gene for counterselection in *S. lividans*.

Our vectors exploiting the cytosine deaminase-based counterselection method include the aminoglycoside resistance gene from the pFDNeo-S plasmid for positive selection of the first homologous recombination event. To avoid duplication of DNA segments inside the vector, the synthetic promoter present in the NeoS cassette (8), already used in *codAS* construction, was replaced by the promoter of the *aacC4* gene, known to be active in *E. coli* and actinomycetes (2), resulting in the *neoA* gene. *S. lividans* TK24 harbouring a single copy of *neoA* was resistant to 600 μg ml^{-1} of Kan in minimal medium (Table 3).

Gene disruption was first attempted for the *S. lividans* homolog of the SCO2657 gene (*2657h*) encoding a putative ROK-family protein (46), localized in an area well conserved among *S. coelicolor* A3(2) and *S. lividans* (26). The upstream and downstream disruption cassettes were amplified from *S. lividans* TK24 genome using primers based on the genome sequence of *S. coelicolor* A3(2) (1). The amplified fragments were cloned into pMP101 on both sides of the *neoA* gene (Figure 1A) and the resulting plasmid pMP101-Ω2657h was introduced into *S. lividans* TK24 by transformation. A Kan^R colony, presumably resulting from a single crossover was picked up. The spores obtained from this colony were plated on minimal

medium with Kan (250 μg ml^{-1}) and 5-FC (200 μg ml^{-1}). 5-FC^R Kan^R colonies, representing putative second crossover recombinants, appeared at a 10^{-3} frequency.

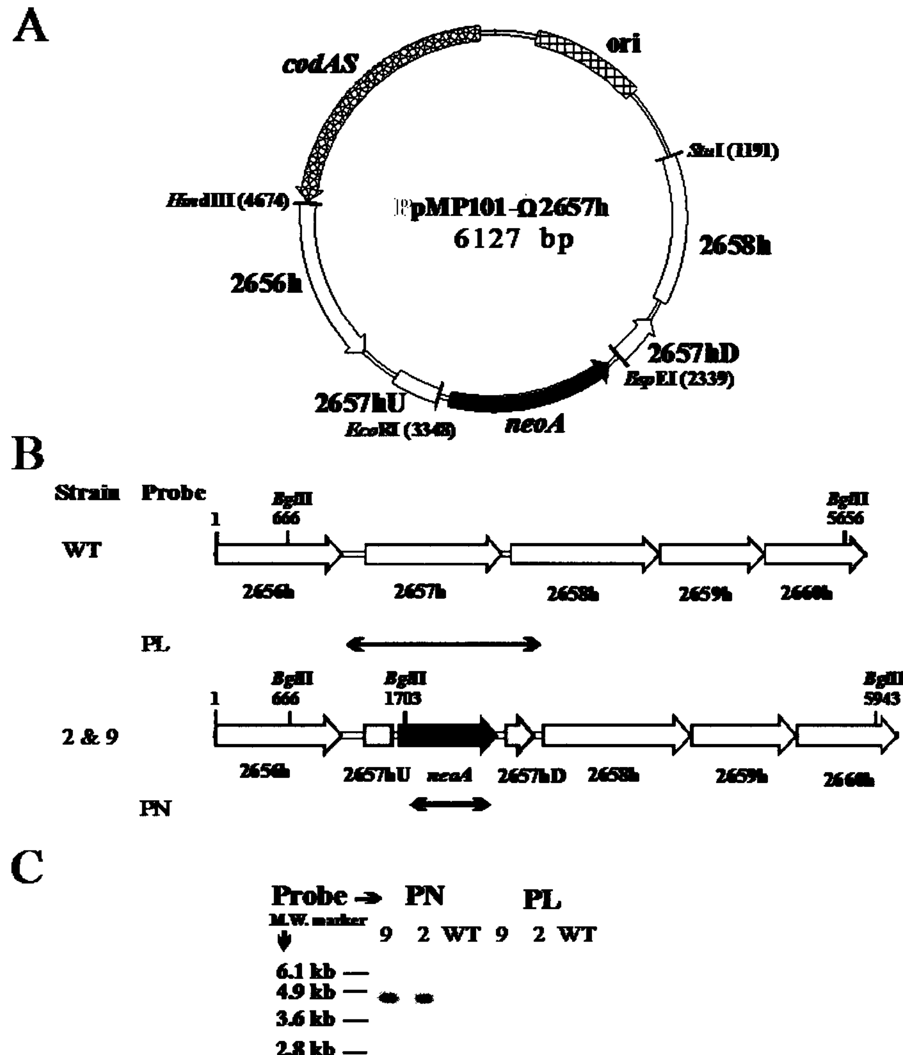

Figure 1: Insertional inactivation of the *S. lividans* SCO2657 homolog gene (2657h) via double crossing over.A) Map of the pMP101 vector derivative (pMP101-Ω2657h) harbouring *S.lividans* genome PCR-amplified fragments for homologous recombination. The upstream fragment was inserted between *Hin*dIII and *Eco*RI sites of pMP101; the downstream fragment between *Bsp*EI and *Stu*I sites. B) Schematic diagram showing the wild type (WT) genomic section of *S. lividans* and its configuration after genomic disruption of *2657h* by double crossing over in two independent colonies (2 & 9). 2656h, 2658h, 2659h and 2660h represents *S. lividans* homologs of SCO2656, SCO2658, SCO2659 and SCO2660 genes respectively.

The PN and PL denote DNA fragments used as probes in Southern blotting. PN probe is internal to the *neoA* gene and the PL probe covers the entire *2657h* gene together with flanking sequences. C) Southern blotting analysis of *2657h* gene area before (WT) and after (2 & 9) gene disruption. Genomic DNA from the host strain (WT) or from randomly picked colonies resistant to 5-FC (2 & 9) was digested with *Bgl*II, subjected to electrophoresis and blotting and hybridized with probes PN or PL.

Figure 1B summarizes the recombination events between pMP101-Ω2657h and the *S. lividans* chromosome. Genomic DNA was extracted from two, randomly selected second-crossover 5-FC^R Kan^R clones (2 and 9; Fig. 1B) and digested with *Bgl*II for analysis by Southern blotting and hybridization. Expected hybridization patterns were observed (Figure 1C), indicating the successful disruption of the ROK protein-encoding gene with insertion of the *neoA* cassette. A parallel Southern blotting analysis has been done by *Bsi*WI digestion, confirming the expected configuration of the disrupted gene (not shown).

The mutated gene *codASM* increases sensitivity to 5-FC.

The mutant D314A of CodA was obtained by alanine-scanning mutagenesis (34) in an attempt to identify enzyme forms with altered substrate specificity. The mutated enzyme has a 17-fold lower activity against native cytosine while its activity toward 5-FC is twofold higher (34). A recent work demonstrated the usefulness of this mutant for tumour therapy *in vivo* when expressed in a high G+C microorganism, *Bifidobacterium longum* (18). We thus introduced the D314A mutation into the *codAS* gene, obtaining *codASM. S. lividans* TK24 harbouring one integrated copy of *codASM* (TK24[pMGi-codASM]) was 10-times more sensitive to 5-FC than TK24 *codAS* (TK24[pMPi-codAS]) and 40-times more sensitive than the native TK24 (Table2), while its MIC for 5-FU remained under 0.1 μg ml^{-1} (Table 3). Thus, the use of *codASM* allows performing counterselection and select 5-FC^R *S. lividans* strains at low 5-FC concentrations, similar to those routinely used with bacterial species naturally possessing cytosine deaminase activity, such as *E. coli* (29).

New vectors for gene disruption and replacement exploiting *codAS*-based counterselection.

To increase the versatility of *codAS*-based vectors, we replaced the rudimentary cloning site of pMP101 with polylinkers responding to the following criteria: 1) they are among the rarest sites in actinobacterial genomes 2) they produce cohesive ends after digestion and 3) they contain a mix of G/C and A/T pairings. We thus analyzed several complete genome sequences from actinobacteria to determine the rarest restriction sites meeting our criteria (Table 4). Polylinker *Hind*III-*Eco*RI was introduced between *codAS* and *neoA*, while polylinker *Xba*I-*Pvu*I is localized downstream from *neoA* (Figure 2 and Figure 3). Both polylinkers will accommodate DNA fragments in gene disruption procedures involving insertion of *neoA* into the inactivated gene, while only the polylinker *Xba*I-*Pvu*I will be used for cloning the fragment(s) involved in gene replacement, including in-frame unmarked deletions. The vector pMG201M is identical to pMP201 except that *codAS* has been replaced with *codASM* (Figure 3).

Table 4: Occurence of pMP-vectors polylinker restriction sites in completely sequenced actinobacterial genomes.

Resctriction site	Sequence	SCO	SAV	SER	MSM	NFA
*Avr*II	C/CTAGG	196	229	135	104	100
*Xba*I*	T/CTAGA	63	69	48	83	33
*Nhe*I	G/CTAGC	115	170	262	144	137
*Mfe*I	C/AATTG	85	120	118	331	181
*Bsr*GI*	T/GTACA	609	618	303	666	341
*Bsp*EI*	T/CCGGA	2788	2859	2877	1880	1597
*Eco*RI	G/AATTC	318	574	520	1197	1402
*Nsi*I	ATGCA/T	121	249	150	268	113
*Afl*II	C/TTAAG	27	49	32	29	13
*Spe*I	A/CTAGT	41	54	80	61	37
*Hind*III	A/AGCTT	100	148	170	183	53

Complete genomic sequences have been analyzed for the occurrence of the respective restriction sites. SCO: *Streptomyces coelicolor* A3(2), GenBank file NC_003888; SAV: *Streptomyces avermitilis* MA-4680, NC_003155; SER: *Saccharopolyspora erythraea* NRRL 2338, NC_009142; MSM: *Mycobacterium smegmatis* MC^{2}155, MC_008596; NFA: *Nocardia*

farcinica IFM 10152, NC_006361. (*) These sites were already present in segments adjacent to the newly introduced polylinker.

We also constructed two conjugative vectors: pMP301 resulted from the addition of *oriT* to pMP201 allowing conjugative transfer from *Escherichia coli* to several actinobacteria (36), while pMP302 harbours an ampicilin resistance gene (*bla*) for selection in *E. coli* conjugative donor strains already resistant to aminoglycosides (Figure 2 and Figure 3).

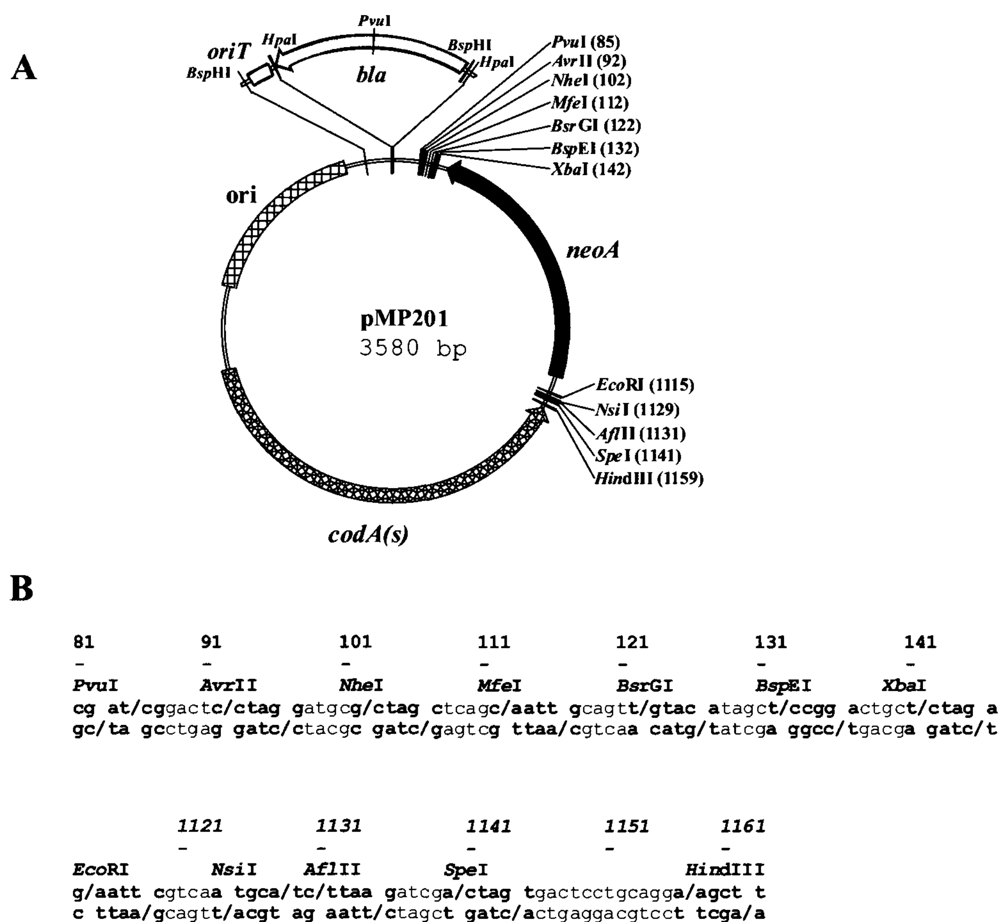

Figure 2: Elements of pMP201 vector and derivatives A) Map of the pMP201 vector with polylinkers *Xba*I-*Pvu*I and *Hin*dIII-*Eco*RI. *neoA* – aminoglycoside resistance gene with promoter from *aacC4* gene; *codAS* – synthetic version of the cytosine deaminase gene; ori – origin of replication for *E. coli*. The position of *oriT* (origin of conjugative transfer) present in pMP301 and pMP302 and *bla* (ampicilin-resistance gene) present in pMP302 is also shown. Note that the *Pvu*I site in the polylinker is not unique in derivatives containing the *bla* gene. B) DNA sequence of polylinkers in pMP201 and derivatives (coordinates are from pMP201 sequence).

As a further example of use of the cytosine deaminase gene as a negative selection marker, the vector pMG201M was used for disruption of the chitosanase gene (*csnA*) in *Streptomyces lividans* TK24. This gene is almost identical with the SCO0677 gene from *Streptomyces coelicolor* A3(2), shown to encode a chitosanase secreted by the Tat-secretion pathway (31). Amplified genomic fragments were cloned into pMG201M on both sides of the *neoA* gene and the resulting plasmid pMG201M-Ω*csnA* was introduced into *S. lividans* TK24 by transformation. A Kan^R colony, presumably resulting from a single crossover, was picked up. Spores obtained from this colony were plated on minimal medium with Kan (250 μg ml^{-1}) and 5-FC (50 μg ml^{-1}). 5-FC-resistant colonies appeared at a $7x10^{-3}$ frequency. Fragments of the expected length were observed by on-colony PCR performed on two independent $5\text{-}FC^R$ colonies, indicating successful disruption of the *csnA* gene (data not shown).

The conjugative vector pMP302 has been used to obtain replacement of the *S. lividans* homolog of the SCO2657 gene by its version interrupted by an unmarked in-frame deletion. The upstream and downstream disruption cassettes were amplified from *S. lividans* genome and cloned as *Bsp*EI-*Nhe*I-*Avr*II fragment in pMP302 (Figure 3). After conjugation of *S. lividans* spores with *E. coli* ET12567 (pUZ8002) (pMP302-F2657h), one single-crossover Kan^R clone was obtained with each cell: spore ratio (1:1 and 40:1) resulting in a recombination frequency of $5x10^{-7}$. In the counterselection step on plates with 200 μg 5-FC ml^{-1}, plating of 20000 spores yielded 18 5-FC-resistant colonies, resulting in a second crossover frequency of approximately 10^{-3}. Out of 12 purified clones, 11 regained the original gene configuration while one harboured the in-frame deletion which was then verified by PCR and Southern blotting (data not shown). Figure 3 summarizes the construction of the various vectors described in this work.

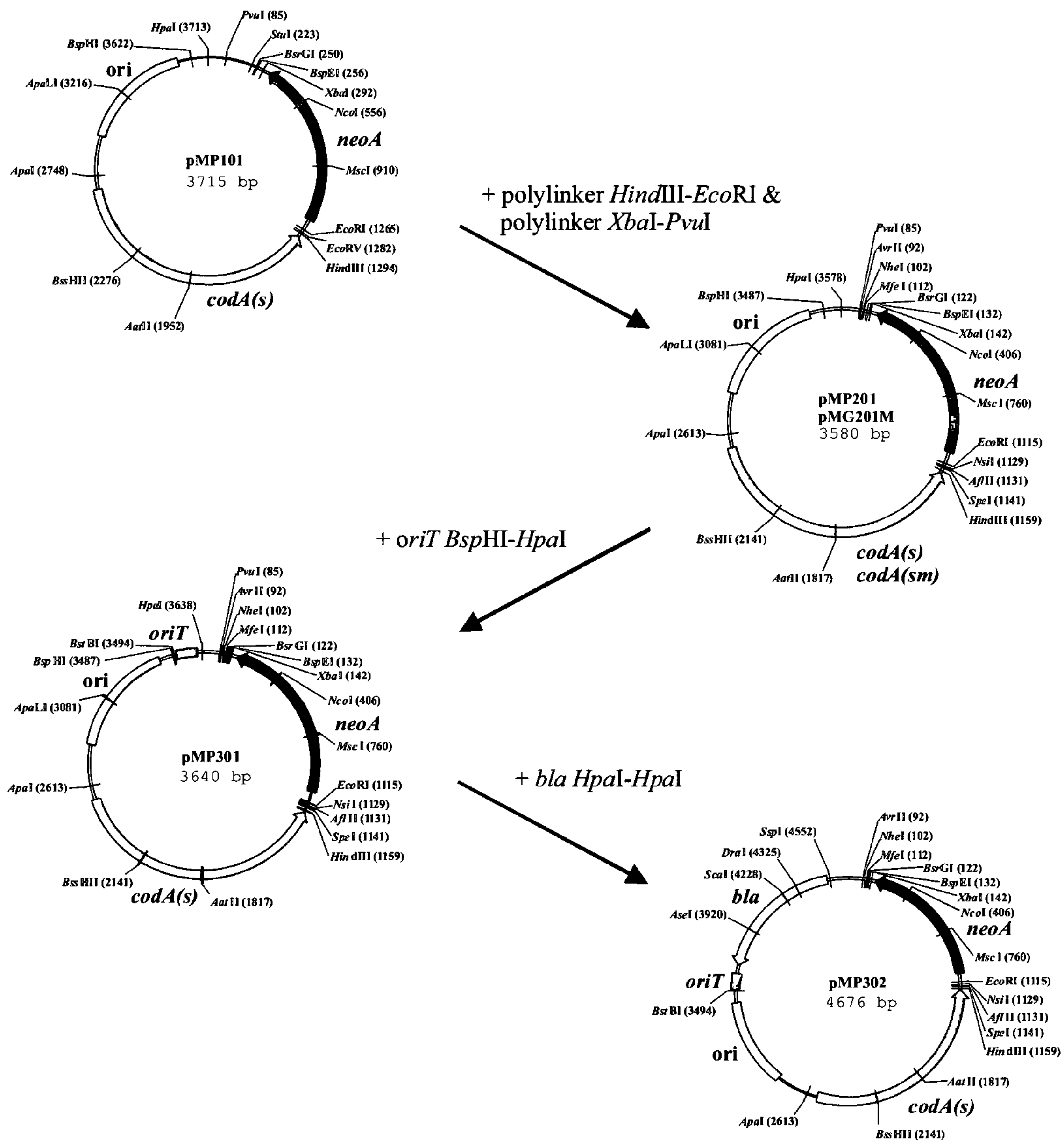

Figure 3: Restriction maps of the vectors constructed in this work.
All unique restriction sites are shown with their coordinates in the respective plasmids. pMP101 – first generation vector (*neoA*; *codAS*). pMP201 – second generation vector (*neoA*; *codAS*) derived from pMP101 by addition of polylinkers; pMP301 – conjugative version of pMP201 (*neoA*; *codAS*; *oriT*); pMP302 – derivative of pMP301 obtained by addition of the ampicilin resistance gene *bla*. pMG201M was obtained by replacement of the *codAS* gene by the single point D314A mutant, *codASM.* Its restriction map is identical to that of its parent plasmid.

Conclusion.

We have shown that the cytosine deaminase gene can be efficiently used as a negative selection marker for directed gene disruption and replacement in *Streptomyces* and possibly in other actinobacteria, by facilitating the construction of stable, double-crossover mutants in the gene of choice. The method may be used with any actinobacterial species naturally resistant to 5-FC while sensitive to 5-FU without it being necessary to introduce specific mutations into the strain beforehand. For some species which are resistant both to 5-FC and 5-FU (Table 2), a phenotype resulting probably from inefficient transport of exogenous pyrimidines, the addition of a cytosine permease gene to the vectors could result in improved sensitivity to 5-FC, allowing the application of our method. The CodA-based technology should be a valuable addition to the actinobacterial genetic toolbox.

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CHAPTER III

3.0 Biochemical and physiological studies of the chitosanase SCO0677 from *Streptomyces coelicolor* A3(2).

Chitosanases, enzymes catalyzing the hydrolysis of glycoside links in chitosan, are found in many organisms, including bacteria, fungi, and plants. In the last three decades, chitosanases have been intensively studied as tools for biotechnological transformation of chitosan. Lately, chitosanases and chitosan are being investigated for their role in the maintenance of the ecological balance in nature. In the first chapter, we showed that the expression of a heterologous chitosanase in *E. coli* (naturally devoid of chitosanase activity), conferred a protection for the expressing host against the bactericidal effect of chitosan. Next we were interested to see the effect that the chitosanase production could have on the survival, in the presence of chitosan, of an organism from the streptomycetes group, naturally possessing a chitosanase activity.

The studies presented in this chapter can be divided into two parts. The first part is dedicated to the biochemical characterization of the chitosanase CsnA from *Streptomyces coelicolor* A3(2) while the second part has a more fundamental character and consists in the study of the phenotypical effect of production of this chitosanase in *Streptomyces lividans* TK24.

The gene SCO0677 from *Streptomyces coelicolor* A(2), encoding the chitosanase CsnA, was cloned, the protein was produced by the heterologous host *Streptomyces lividans* 10-164 and the biochemical properties of CsnA were determined. Based on its primary structure, CsnA is highly similar to other chitosanases from actinomycetes such as CsnN174 from *Streptomyces*

sp. N174 and CsnN106 from *Kitasatospora* sp. N106 and, therefore, it belongs to the GH46 family. Interestingly, the CsnA is secreted *via* the Tat-dependent pathway, while CsnN174 has a Sec-dependent N-terminal signal peptide. Here, we compared the rate of CsnA secretion *via* Tat-pathway to that *via* the Sec-pathway. These studies allowed us to better understand chitosanase secretion in streptomycetes.

Streptomyces coelicolor A(2) is closely related to *Streptomyces lividans* TK24, both of them belonging to the *Streptomyces violaceoruber* species. Hence, we took advantage of the close genetic relatedness between the two strains and we have disrupted the chitosanase gene in *S. lividans* TK24 (work presented in Chapter 2). In this chapter we analysed the effect of chitosanase gene disruption on the survival of the mutant strain, in the presence of chitosan. *In vivo* experiments presented in this chapter show that, in the presence of chitosan, growth of the mutant strain as well as its ability for xylose uptake were impaired compared to the wild type strain or to a chitosanase over-producing strain. The results presented in this chapter suggest that chitosanases may play an important role in the protection of bacterial cells against the antimicrobial effect of chitosan.

The results obtained in this work are presented in details in the next enclosed article. This article will be submitted to Biochemistry and Cell Biology:

Ghinet, M.G., Roy, S., Lacombe-Harvey, M.-È., Morosoli, R. and Brzezinski, R. (2009). Chitosanase from *Streptomyces coelicolor* A3(2): biochemical properties and role in protection against antibacterial effect of chitosan.

Several authors contributed to the realization of this work. Their contribution is described above. Dr. S. Roy performed the thermal unfolding experiments for CsnA and CsnN174. M.-È

Lacombe-Harvey optimized the protocol for the kinetic assays. I amplified the *csnA* gene from the genomic DNA of *Streptomyces coelicolor* A3(2) with specific PCR primers. Once the *csnA* sequence was confirmed by sequencing, the gene was cloned in the vector pIAF*csn*A0624 in Dr. R. Morosoli's laboratory. Furthermore, Dr. R. Morosoli has provided us with the *S. lividans* 10-164 and *S. lividans* 10-164 Δ TatC strains. I constructed the plasmid pIAF*csn*ASec and performed all the experiments concerning chitosanase secretion presented in this article. I also optimized the purification protocol for CsnA. I performed all the experiments related to the biochemical characterization of this protein. I confirmed the *csnA* sequence from *S. lividans* TK24, by sequencing. I also purified the CsnA from *S. lividans* for further analysis (N-terminal sequencing and enzymatic activity). Moreover, I determined the minimal inhibitory concentration of chitosan and realized the experiments of xylose uptake in liquid media with chitosan for the strain described above. Together with Dr. Brzezinski, I analysed the results and wrote the manuscript.

3.1 CHITOSANASE FROM *STREPTOMYCES COELICOLOR* A3(2): BIOCHEMICAL PROPERTIES AND ROLE IN PROTECTION AGAINST ANTIBACTERIAL EFFECT OF CHITOSAN

Chitosanase from *Streptomyces coelicolor* A3(2): biochemical properties and role in protection against antibacterial effect of chitosan

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ABSTRACT

Chitosan, an *N*-deacetylated derivative of chitin, has attracted much attention as an antimicrobial agent against fungi, bacteria and viruses. Chitosanases, the glycoside hydrolases responsible for chitosan depolymerisation, are intensively studied as tools for biotechnological transformation of chitosan. In this study, the chitosanase CsnA from *Streptomyces coelicolor* A3(2) was purified and its biochemical properties determined. CsnA belongs to the GH46 family of glycoside hydrolases. However, it is secreted efficiently by the Tat translocation pathway despite its similarity to the well studied chitosanase from *Streptomyces* sp. N174 which is preferentially secreted through the Sec pathway. We further assessed the role of CsnA as a potential protective enzyme against the antimicrobial effect of chitosan. A *Streptomyces lividans* TK24 strain, in which the chitosanase gene was inactivated by gene disruption, was more sensitive to chitosan than the wild type strain or a strain overproducing chitosanase. This is the first evidence (obtained through gene inactivation) for the involvement of chitosanases in the protection of the bacteria against the antimicrobial effect of chitosan.

INTRODUCTION.

Chitosan is a polysaccharide of β-1,4-linked glucosamine residues, partially substituted with *N*-acetyl groups. In nature, this polymer is found in the cell wall of *Zygomycetes*, a group of fungi comprising opportunistic human pathogens (*Mucor*) and phytopathogens (*Rhizopus*) (Tan *et al*., 1996), as well as in the cell wall of *Chlorella*, a green algae (Kapaun and Reisser, 1995).

Chitosanases (EC 3.2.1.123) are enzymes that hydrolyse the β-1,4-linkages in partly acetylated chitosan. They have been found in chitosan-utilizing microorganisms such as bacteria and fungi as well as in *Chlorella* viruses (Sun *et al*., 1999; Boucher *et al.,* 1992; Saito *et al*., 2009; Park *et al*., 1999, Alfonos *et al*, 1992, Wang *et al*., 2008). Chitosanase activities have been also detected in plants (Osswald *et a*l., 1993).

Streptomyces coelicolor A3(2), the model actinomycete for genetic studies, belongs to the *Streptomyces violaceoruber* species, along with *Streptomyces lividans,* a popular host for recombinant proteins production (Hatamo *et al*., 1994). For many years, *S. lividans* and *S. coelicolor* have been distinguished mainly by the inability of *S. lividans* to produce actinorhodin, to degrade agar (Kieser *et al*., 2000), to methylate its own DNA or to restrict exogenous methylated DNA (Choulet *et al*., 2006). The 16S rRNA sequences of these strains share >99.5% identity. Furthermore, the comparison of the physical maps of their chromosomes revealed a common structure (Leblond *et al*., 1993). In 2007, Jayapal *et al.* performed an exhaustive microarray-based comparison of these strains and they noted that 90% of *S. coelicolor* genes are conserved remarkably well in *S. lividans*. However, they identified five large *S. coelicolor* genomic islands (>25 kb) and 18 smaller islets that are absent in the *S. lividans* chromosome. The absence of the genes comprised in those regions was the main reason for the differences between the two strains described above (Kieser *et al*., 2000; Choulet *et al*., 2006).

For years, *Streptomyces lividans* TK24 was thought to lack a chitosanase activity. When grown in a liquid medium containing chitosan as sole carbon source, no chitosanase activity could be detected (Neugebauer *et al.*, 1991). Furthermore, when tested on a solid chitosanase detection medium (Boucher *et al.*, 1991), *S. lividans* TK24 gave a very weak degradation zone. Thus, the low activity of this strain against chitosan was attributed to the production of chitinases having low affinity for chitosan (Masson *et al.*, 1995). In this aspect, *S. coelicolor* A3(2) is very similar to *S. lividans* TK24 as it produces very low amounts of chitosanase when grown on chitosan.

More recently, the complete genome sequence of *Streptomyces coelicolor* A3(2) became available (Bentley *et al.*, 2002). It revealed a whole array of genes/proteins dedicated to the hydrolysis and metabolism of chitin and chitosan. Surprisingly, two genes, SCO0677 (*csn*A) and SCO2024 (*csn*B), encode putative chitosanases belonging to the family 46 of glycoside hydrolases. The *csn*A gene encodes a 280-amino-acid protein with an N-terminal signal peptide of 44 amino acids. Analysis of the signal peptide sequence using the TATscan program revealed that CsnA is a protein secreted mainly by the Tat system (Li *et al.*, 2005), which was confirmed by functional studies. Thus, CsnA became the first GH46 chitosanase to be secreted by the Tat-dependent pathway.

As a follow-up of the previous secretion study, we report here the biochemical characterization of the chitosanase CsnA from *Streptomyces coelicolor* A3(2). Furthermore, disruption of *csn*A in *S. lividans* TK24 resulted in an increased susceptibility of the mutant strain towards the toxic effect of chitosan. This brings into perspective a new biological role for chitosanase in the protection against the antimicrobial effect of chitosan. This is the first report bringing genetic evidence (obtained through gene inactivation) for the protective role of a chitosanase against the bactericidal effect of chitosan.

Materials and Methods.

Bacterial strain, plasmids and culture media.

Streptomyces lividans 10-164 and the isogenic *tatC* mutant were used as hosts for recombinant plasmids (Li *et al.*, 2005). The pIAF*csn*A0624 plasmid harbours the mature part-encoding segment of *csn*A under the control of the promoter of xylanase A gene from *S. lividans* and the signal peptide sequence of the SCO0624 (TAT-dependent) gene from *Streptomyces coelicolor* A3(2) (Li *et al.*, 2005). The pIAF*csn*ASec plasmid, a derivative of the pIAF*csn*A0624 plasmid, was obtained by replacement of the signal peptide sequence of the SCO0624 gene with the Sec-dependent signal peptide sequence of the cellulase A gene (Morosoli *et al.*, 1997) from *S. lividans* as a *Hin*dIII-*Kpn*I fragment. The strains *Streptomyces lividans* TK24 (Kieser *et al.*, 2000), *Streptomyces lividans* TK24 Δ*csn*A::*neo*A and *S. lividans* TK24 [pMPi-csnN106] have been described previously (Dubeau *et al.*, 2008; Dubeau *et al.*, 2009).

Enzyme production and purification.

CsnA was produced as an extracellular protein by the heterologous host *Streptomyces lividans* 10-164 as described previously (Roy *et al.*, 2007). The chitosanase was purified following a simple two-step procedure. All the purification steps were done at 4°C. The culture supernatant fluid was adjusted to pH 7.5 with Tris-Base and loaded on a Q-Sepharose Fast Flow (GE Healthcare Bio-Sciences, Baie d'Urfé, QC, Canada) column equilibrated with Tris-HCl 20 mM buffer pH 7.5. The chitosanase was eluted at 0.3 M NaCl and the chosen fractions were dialysed against a solution of $MgCl_2$ 1 mM, and then loaded on a Hydroxylapatite Fast Flow (Calbiochem, Darmstadt, Germany) column pre-equilibrated with a solution of $MgCl_2$ 1 mM. Chitosanase was eluted with $MgCl_2$ 1 M. The purified chitosanase was dialysed against 50 mM sodium acetate buffer pH 5.5. Purity was evaluated by 12% SDS-PAGE (Laemmli 1970). Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard. Protein concentration of purified chitosanase was determined by UV absorbance (280 nm) using a molar extinction coefficient of 26,213 M^{-1}

cm^{-1}. For N-terminal sequencing, the protein was subjected to 12% SDS-PAGE in 25 mM Tris/ 250 mM glycine/ 0.1% SDS buffer. After migration, the protein was transferred to a PVDF membrane (Millipore, Billerica, MA, U.S.A.) using 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) / 20% methanol as transfer buffer (Findlay and Geisow, 1989). The N-terminal protein sequence was determined by Edman degradation on a Procise Clc sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Isoelectric point determination.

4.3 µg of chitosanase were mixed with 40 µl of the sample buffer (9 M urea/ 2 % CHAPS/ 0.8% Pharmalyte/ 15 mM DTT), and then loaded on pH 3-10, 7 cm IPG strips (GE Healthcare). After a 12h rehydration at room temperature, IEF (isoelectric focusing) was carried out using the IPGphor IEF System (GE Healthcare) according to the manufacturer's instructions. Strips were equilibrated for 15 min. while shaking in 50 mM Tris, pH 8.8 / 2% SDS / 6 M urea / 30% glycerol containing 1% DTT buffer followed by 15 min. incubation in the same buffer containing 4% iodoacetamide. Strips were then transferred onto a 12% gel for a SDS-PAGE conducted for 2h 30min. at 66V and 22A. Finally the gel was colored with Coomassie Blue and image analysis was carried out using Phoretix 2D Image Analysis Software (Nonlinear Dynamics).

Enzyme assays.

Chitosanase activity was determined using the reducing sugar assay (Lever *et al.*, 1972), as modified by Schep *et al.* (1984). Chitosan (79% deacetylated, Sigma-Aldrich; St-Louis, MO, USA) was dissolved at a final concentration of 0.8 mg/ml in 50 mM sodium acetate pH 4.1 and incubated with the enzyme for 10 min. at 37°C. The reaction was stopped by addition of the *p*-hydroxybenzoic acid reagent prior to final incubation for 20 min. at 99.8°C. Absorbance was measured at 405 nm. D-glucosamine was used as standard. To determine the optimal pH, the chitosanase was incubated for 10 min. at 37°C in the presence of 0.8 mg/ml chitosan dissolved in sodium acetate buffer solutions with pH values varying from 3.6 to 5.5. The

optimal temperature of CsnA was determined by incubating the enzyme for 10 min. in a 50 mM sodium acetate buffer solution (pH 4.1) containing 0.8 mg/ml chitosan at temperatures varying from 25°C to 75°C. Three replicas were done for each condition.

For kinetic assays, 0.2 μg (5.4 mU) of purified protein were mixed with 0.4 ml of reaction mixtures containing eight different concentrations (0.02 - 0.2 mg ml^{-1} in sodium acetate buffer 100 mM pH 4.1) of chitosan. Eight replicas were made for each chitosan concentration. *K*m and k_{cat} values were calculated using the non linear least-square fitting procedure for Michaelis-Menten equation in PRISM software (version 5.0 for Windows, San Diego, CA, U.S.A.).

Proteolytic activity was determined with azocasein (Aretz and Riess, 1989). One protease unit was defined as the amount of enzyme required to increase OD_{366} by 0.01 in 30 min. at 50°C.

Substrate specificity.

Enzyme activity was measured against mannan, methyl cellulose, polygalacturonic acid, laminarin, β-(1,3)(1,4)-glucan, cellulose, chitin, and birchwood xylan (Sigma-Aldrich), pachyman and pustulan (Calbiochem, Darmstadt, Germany). All these substrates were prepared at a final concentration of 1 mg/ml in 50 mM sodium acetate pH 4.1 and incubated with the enzyme for 3h at 37°C. Activity was estimated by the reducing sugar assay.

Thermal unfolding of chitosanase CsnA.

Chitosan (85% deacetylated, Marinard Biotech, (Rivière-au-Renard, QC) was further purified by sequential precipitations as described (Roy *et al.*, 2007). The lyophilized product was used to prepare 5 g/l solutions in sodium acetate buffer (100 mM), pH of 4.1 or 5.5. Thermostability of chitosanase, as determined by protein folding state, was studied via intrinsic tryptophan fluorescence. Thermal unfolding analyses were performed on a Shimadzu RF-1501

(Shimadzu, Kyoto, JP.) spectrofluorimeter equipped with a Precision thermometer model 460 YSI (Yellow Springs, OH) as described (Roy *et al.*, 2007).

Determination of minimal inhibitory concentration (MIC) of chitosan.

Chitosan polymers of intermediate molecular mass (M_n of 3 or 10 kDa) were prepared from Sigma chitosan (DA = 21%), by partial hydrolysis with endo-chitosanase (Boucher *et al.*, 1992). Chitosan (40 g/l) was dissolved in 0.2 M acetic acid, mixed with chitosanase (0.125 U ml^{-1}) and incubated for 10 min. at 37°C. The reaction was stopped by 30 min. of heating in boiling water. Number average molecular mass (M_n) was estimated by a reducing end assay (Lever 1972). The partly hydrolyzed chitosan was precipitated with ammonium hydroxide, and recovered by centrifugation. The pellet was frozen overnight at -80°C and lyophilized for 48 hours. Finally, the chitosan was prepared as a stock solution of 50 g/l in 0.7 mM HCl and sterilized by filtration. MICs were determined by the agar dilution method (CLSI, 2006). Minimal agar medium ($MgSO_4$ 0.2 g/l, $CaCl_2$ 0.02 g/l, KH_2PO_4 1 g/l, K_2HPO_4 1 g/l, $FeCl_3$ 0.05 g/l and NaCl 2.5 g/l) was supplemented with glucose 1 g/l, $(NH_4)_2SO_4$ 1 g/l and yeast extract 1 g/l. MICs were determined on a series of minimal agar medium plates containing 0, 0.05, 0.075, 0.08, 0.1 or 0.2 g/l of 10 kDa chitosan. Suspensions of spores of *S. lividans* TK24, *S. lividans* TK24 Δ*csn*A::*neo*A or *S. lividans* TK24[pMPi-csnN106] were diluted to 2×10^3cfu ml^{-1}, spotted on the agar plates and incubated at 30°C for 72.

Xylose uptake in liquid media with chitosan.

Spores of S. *lividans* TK24, *S. lividans* TK24 Δ*csn*A::*neo*A or *S. lividans* TK24[pMPi-csnN106] were inoculated into Tryptic Soy Broth (TSB) medium and grown for 72h at 30°C with shaking (250 rpm). Mycelia were harvested by low speed centrifugation and then inoculated into medium M14 (Pagé *et al.*, 1996) supplemented with xylose 5 g/l and 0 to 1.2 g/l chitosan ($M_n \approx 3$ kDa). 5 ml of respective cultures were collected each 24h. After centrifugation, the mycelial pellet was used for dry weight determination and the supernatant tested for residual xylose level and chitosanase activity. Xylose was determined using the

reducing sugars assay of Lever (Lever *et al.*, 1972). Chitosanase activity was determined with sRBB-C substrate (Zitouni *et al.*, 2010): 100 µl of supernatant were added to 900 µl of a sRBB-C solution of 5 g/l 100 mM sodium acetate buffer pH 4.5 and the mixture was incubated at 37°C. After 2h, the reaction was stopped by the addition of 500 µl of 1.2 M NaOH, prior to 20 min. incubation on ice and 10 min. centrifugation at 13000 rpm. Absorbance was measured at 595 nm and activity (A) was calculated in U/ml using the following empirical formula: $A = (-7.1*DO_{595}^2+21.9*DO_{595})/100$.

RESULTS

CsnA is a member of glycoside hydrolase family GH46.

A BLAST search (version 2.2.20) (Altschul *et al.*, 1990) indicated that the CsnA chitosanase belongs to the family 46 of glycoside hydrolases (data not shown). To visualize the relationships among CsnA and other members of GH46, the catalytic module sequences of chitosanases belonging to this family and for which activities have been confirmed by biochemical studies were aligned using T-Coffee (Notredame *et al.*, 2000) and the resulting data were used to generate an unrooted phylogenetic tree. As shown in Figure 1, the eighteen sequences defined three clusters. The chitosanase CsnA belongs to the same cluster as the well studied chitosanases CsnN174 from *Streptomyces* sp. N174 (Boucher *et al.*, 1992, Boucher *et al.*, 1995, Lacombe-Harvey *et al.*, 2009), CsnN106 from *Kitasatospora* sp. N106 (Masson *et al.*, 1995) and CtoA from *Amycolatopsis* sp. CsO-2 (Saito *et al.*, 2009). However, CsnA shows the highest similarity to the putative chitosanase SAV2015 from *S. avermitilis* MA-4680. This aspect will be further discussed in the Discussion section.

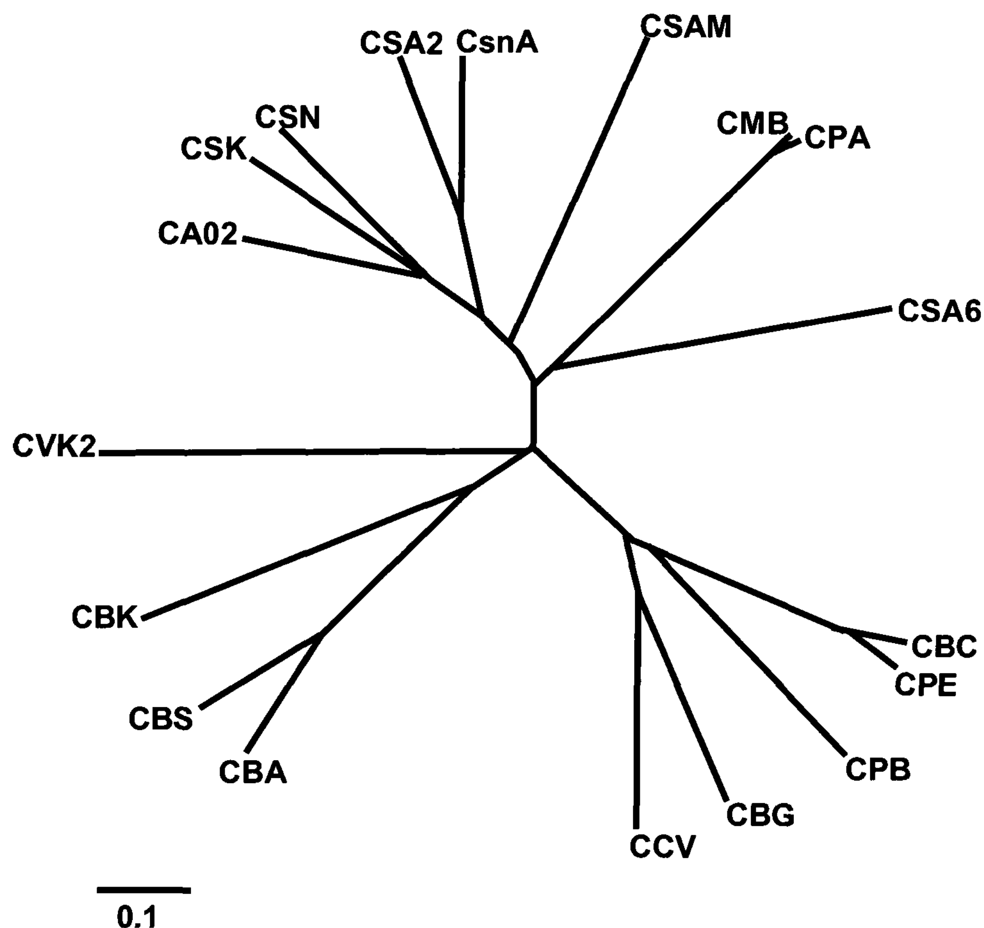

Figure 1: Phylogenetic analysis of primary sequences of GH46 family members.
The sequences of the catalytic modules of the respective proteins originate from the following database entries: CSN, *Streptomyces* sp. N174 (CsnN174, P33665); CSK, *Kitasatospora* sp. N106 (CsnN106, P48846); CA02, *Amycolatopsis* sp. CsO-2 (Q9LBG4); CBA, *Bacillus amyloliquefaciens* MJ-1 (Q0PVM7); CBC, *Bacillus circulans* MH-K1 (P33673), CBK, *Bacillus* sp. KFB-CO4 (Q9RC18); CBS, *Bacillus subtilis* subsp. subtilis str. 168 (O07921); CBG, *Burkholderia gladioli* CHB101 (Q9XDS6); CSA2, *Streptomyces avermitilis* MA-4680 (SAV2015, BAC69726.1); CSA6, *Streptomyces avermitilis* MA-4680 (SAV6191, BAC73902.1), CsnA, *Streptomyces coelicolor* A3(2) (SCO0677, Q9RJ88); CPA, *Pseudomonas* sp. A-01 (Q8KZM5); CSAM, *Streptomyces* sp. AM-7161 (Q7WT07); CCV, *Chromobacterium violaceum* ATCC 12472 (AAQ61593.1); CPB, *Paenibacillus* sp. BH-2005 (Q2PWA1); CPE, *Paenibacillus ehimensis* EAG1 (O24825); CMB, *Microbacterium* sp. OU01 (A7KBW5); CVK2, *Chlorella virus* CVK2 (O12288). The phylogenetic tree was generated using the Phylodendron program (http://iubio.bio.indiana.edu/treeapp/).

Purification and biochemical characterization of CsnA.

Chitosanase CsnA was produced in medium M14 supplemented with xylose 10 g/l as described previously (Roy *et al.*, 2007). A new, time saving purification protocol, in which the size exclusion chromatography step was replaced with a faster hydroxylapatite step, was used (Table 1).

Table 1: Purification of CsnA from a culture of recombinant *Streptomyces lividans* 10-164 pIAFcsnA0624

Step	Protein(mg)	Total activity(units)	Specific activity units/mg	Yield (%)	Purification factor
Crude enzyme	82.2	974.8	11.7	100	1.0
Q-Sepharose	36.0	756.0	21.0	78	1.8
Hydroxyapatite	21.0	571.1	27.1	59	2.3

CsnA was purified to homogeneity as judged by 12% SDS-PAGE and Coomassie Blue staining (Figure 2)

The N-terminal sequence of the purified enzyme was determined to be: ATGLDDPAKKE. However, when analysed using the SignalP 3.0 algorithm (Bendtsen *et al.*, 2004) the predicted cleavage site occurred three residues earlier (AAA↓SARATGLDDPAKKE) than the one determined by N-terminal sequencing. This result led us to assume that CsnA undergoes post-secretory modifications. Thus, the mature protein was composed of 236 amino acids with an approximative molecular mass of 26.2 kDa.

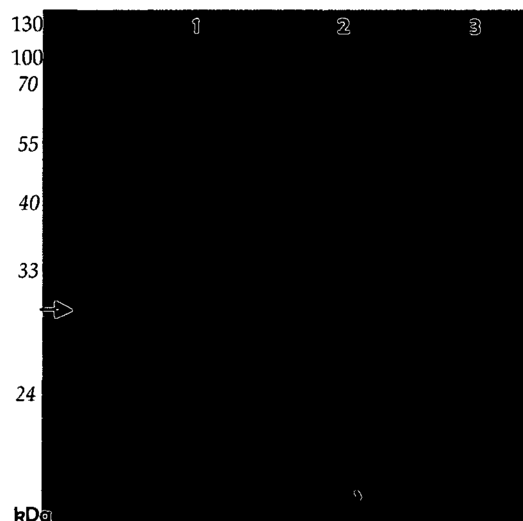

Figure 2: SDS-PAGE (12%) analysis of CsnA. (1), 10 µl of filtrate of culture supernatant; (2), 10 µl of pooled fractions after the Q-Sepharose Fast-Flow step; (3), 5 µl from the pooled fractions after the hydroxyapatite step.

In a study dedicated to secretion mechanisms in *Streptomyces*, Li *et al.* (2005), showed that CsnA is a Tat-dependent protein. By using the TATscan program, a putative Tat motif (SRRTVLA) has been identified in the signal peptide sequence of CsnA. The construction of a fusion gene including the mature part encoding sequence of the CsnA proceeded by the signal peptide of SCO0624 (Tat-dependent) resulted in a 480% increase in chitosanase production (compared to that directed by the wild-type signal peptide) (Li *et al.*, 2005). Furthermore, when the signal peptide of xylanase A (Sec-dependent) was fused to the mature part-encoding sequence of the CsnA a 75% decrease in chitosanase production was observed (compared to the construction carrying the wild-type signal peptide). However, in their study, Li and coworkers did not purify the chitosanase produced by the two pathways for further analysis.

In order to confirm the poor CsnA secretion observed *via* the Sec-dependent pathway, the Sec-dependent signal peptide sequence of xylanase A (from the previous study) was replaced by that of the cellulase A gene from *S. lividans,* in the pIAF vector carrying the mature part encoding segment of SCO0677 gene. The resulting plasmid, named pIAF*csn*ASec, was transformed in *S. lividans* 10-164 and *S. lividans* 10-164 Δ TatC strains. pIAF*csn*A0624 in which the mature part encoding segment of SCO0677 gene was ligated to the Tat-dependent

signal peptide sequence from the SCO0624 gene was also transformed in the same strains. Then we compared the chitosanase levels directed by these two fusion genes in two genetic backgrounds (Figure 3).

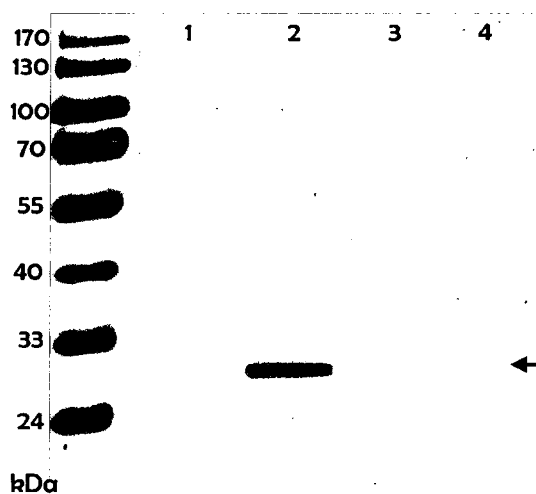

Figure 3: Comparison of recombinant chitosanase production directed by Sec (1 and 3) or Tat (2 and 4) dependent systems, in *S. lividans* 10-164 (1 and 2) and *S. lividans* 10-164 Δ TatC (3 and 4) strains. Samples (10 μl) of supernatants were collected after 24h of culture in M14 medium (with xylose as carbon source) and analyzed by 12% SDS-PAGE followed by Coomassie Blue staining. The chitosanases bands (indicated by the arrow) were identified by their molecular weight as compared to the PageRuler™ Prestained Protein Ladder (Fermentas, Burlington, ON, Canada).

As shown on Figure 3, when fused to a Sec-dependent signal peptide (lane 1), CsnA was poorly secreted (23%) (quantified with ImageQuant, GE Healthcare) compared with CsnA secretion through the Tat-dependent system (100%; lane 2). This low level was not affected by the Δ TatC context (lane 3). Moreover, when the pIAF*csn*A0624 plasmid was used to transform the *S. lividans* 10-164 Δ TatC strain, only very low amounts (8%) of chitosanase were detected in the culture supernatant (lane 4) compared to *S. lividans* 10-164 strain transformed with the same plasmid (100%; lane 2). This indicated that in a Δ TatC background, CsnA secretion was not redirected through the Sec-dependent pathway.

To compare some properties of CsnA secreted by both pathways, the recombinant CsnA secreted by the Sec-dependent system was purified. The N-terminal sequence was determined

to be identical with that of CsnA secreted by the Tat-dependent pathway. More importantly, both chitosanases had the same specific activity (data not shown) indicating that the full length Sec-secreted CsnA recovered after purification was correctly folded in a post-secretory step. It became evident that the Tat pathway is the best suited for CsnA export. Consequently, further biochemical studies were performed with the enzyme secreted by the Tat pathway.

Biochemical properties of CsnA.

The properties of CsnA will be described and compared with those of the well studied chitosanase CsnN174 (Boucher *et al.*, 1992). The optimal reaction conditions and kinetics parameters were determined with 79% *N*-deacetylated chitosan. The optimum pH for CsnA was tested in sodium acetate buffer with pH values ranging from 3.6 to 5.5. Figure 4A shows that CsnA activity remains relatively constant in the studied pH range. However the highest activity for CsnA was observed at a more acidic value (pH 4.1) than for CsnN174 (pH 5.5) (Boucher *et al.*, 1992).

Figure 4: Effect of pH (A) and temperature (B) on CsnA activity. Purified CsnA (2.8 mU/ml) was incubated for 10 min with substrate at the pH and temperature values indicated.

The optimum temperature of CsnA for a 10 min reaction at pH 4.1 was around 50°C (Figure 4B) which was relatively low compared to that for CsnN174 (65°C) (Boucher *et al.*, 1992). For CsnA almost no activity was detected at temperatures above 60°C. The purified CsnA had a specific activity of 27 U/mg which is comparable to 37 U/mg obtained for CsnN174. The

pIs, determined by IEF, differed significantly for both chitosanases: 4.5 for CsnA and 6.5 for CsnN174.

The kinetic parameters were determined under the optimal conditions as described above. CsnA had a K_m of 54 μg/ml and a k_{cat} of 731 min^{-1} compared to a K_m of 29 μg/ml and a k_{cat} of 727.5 min^{-1} determined for CsnN174 (Boucher *et al.*, 1995).

CsnA was highly specific for chitosan degradation. No hydrolysis of mannan, CM-cellulose, microcrystalline cellulose, polygalacturonic acid, laminarin, 1,3-1,4-β-D-glucan, chitin, birchwood xylan, pachyman or pustulan was observed. Within the detection limit of the reducing sugar assay, CsnA activity against chitosan was at least 6000 times higher than for all the other substrates assayed. CsnA was then as specific as CsnN174 for chitosan hydrolysis (Boucher *et al.*, 1992).

CsnA and CsnN174 have very similar specificities towards chitosan substrates with different degree of *N*-acetylation. Both enzymes exhibited maximal activity in the presence of chitosan with 1% degree of *N*-acetylation. Compared to the values of their activities obtained in the presence of Sigma chitosan (79% deacetylated), used in all the experiments described above (values considered to represent 100% of activity), an increase of 44% was observed in the activity for CsnA and 17% in that for CsnN174, respectively. Furthermore, when a chitosan with 10% degree of *N*-acetylation was tested, only a slight increase in their activities was observed: 4% for CsnA and 3% for CsnN174 (compared to the values obtained for the Sigma chitosan).

Effect of pH and chitosan on CsnA thermostability.

Roy *et al.* (2007) have shown that the analysis of thermal unfolding curves by spectrofluorimetry is a reliable method for the evaluation of the thermal stability of GH46 chitosanases. We thus investigated the effect of the pH and chitosan on the thermostability of CsnA and compared it with CsnN174. The effect of pH was assayed at values being optimal

for the enzymatic activities of the respective chitosanases (4.1 and 5.5). Unfolding curves in Figure 5A revealed that at pH 4.1 CsnA is less thermostable than CsnN174 when analyzed in buffer only, while their Tms (transition temperatures) were almost identical at pH 5.5 (52.0°C and 52.5°C respectively; Figure 5B). On the other hand, the gain in thermostability in the presence of substrate (5 g/l) is much greater for CsnA than CsnN174 at both pH values: 18.6°C versus 13.7°C, respectively at pH 4.1 and 19.0°C versus 13.7°C at pH 5.5. A similar effect of stabilization by substrate has been observed at the intermediate pH 4.5 by Roy *et al.* (2007). Our data also show that the thermostability of CsnA is pH-sensitive in the studied range, as illustrated by the shift in Tm of CsnA between pH 4.1 and 5.5 (+7.5°C in the absence of chitosan or +7.9°C in the presence of 5 g/l of chitosan). In contrast, no apparent shift observed for CsnN174 under the same conditions.

pH 4.1 pH 5.5

Figure 5: Thermal unfolding of CsnA (○, ●) and CsnN174 (Δ, ▲) chitosanases at pH 4.1 (A) or pH 5.5 (B) in the absence (open symbols) or presence of 5 g/l chitosan (closed symbols).

***In vivo* studies: assessment of the possible role of CsnA in protection against the antimicrobial effect of chitosan.**

In vivo studies were done with the *S. lividans* TK24 strain, considered, by several authors, as member of the same species as *S. coelicolor* A3(2). The lack of agarolytic activity in the former (Kieser *et al.*, 2000) facilitated the interpretation of the experiments performed on agar plates. We first cloned and sequenced the CsnA ortholog of *S. lividans* (GeneBanK accession no GQ438786). It turned out that this gene differed only by seven point mutations from that of

S. coelicolor A3(2) (not shown). Translated into an amino acid sequence, four of these mutations were silent; one was present in the signal peptide and thus did not influence the sequence of the mature chitosanase while the other two did not affect amino acids essential for chitosanase function (Figure 6). We also purified the chitosanase from *S. lividans* and its N-terminal sequencing revealed that this protein has the same N-terminus as the CsnA chitosanase used for the biochemical studies (data not shown).

```
S.liv      VRHPFRDPARPAGPSRRTVLAMACASLATVPLFTPSRAAAASARATGLDDPAKKEIAMQL  60
S.coel     MRHPFRDPARPAGPSRRTVLAMACASLATVPLFTPSHAAAASARATGLDDPAKKEIAMQL  60
           :***********************************:***********************

S.liv      VSSAENSSLDWKAQYRYIEDIGDGRGYTAGIIGFCSGTGDMLDLVELYGQRSPGNVLAPY  120
S.coel     VSSAENSSLDWKAQYRYIEDIGDGRGYTAGIIGFCSGTGDMLDLVELYGERSPGNVLAPY  120
           *************************************************:**********

S.liv      LPALRRVDGSDSHEGLDPGFPDDWRRAADQDPQFRRAQDDERDRVYFDPAVRRGKEDGLR  180
S.coel     LPALRRVDGSDSHEGLDPGFPDDWRRAADQDPQFRRAQDDERDRVYFDPAVRRGKEDGLR  180
           ************************************************************

S.liv      TLGQFAYYDAMVMHGDGGGLGFGSIRERALGRARPPAQGGDEVAYLHAFLDERVWAMKQE  240
S.coel     TLGQFAYYDAMVMHGDGGGLGFGSIRERALGRARPPAQGGDEVAYLHAFLDERVWAMKQE  240
           ************************************************************

S.liv      EAHSDTSRVDTAQRVFLNEGNLDLEPPLDWHVYGDAYHIG  280
S.coel     QAHSDTSRVDTAQRVFLNEGNLDLEPPLDWHVYGDAYHIG  280
           :***************************************
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Figure 6: Alignment of primary sequences of the chitosanases from *S. lividans* TK24 and *S. coelicolor* A3(2). Amino acids affected by the point mutations are boxed. The arrow indicates the signal peptide cleavage site determined by N-terminal sequencing.

The *S. lividans* CsnA is thus almost identical to the enzyme we have studied by biochemical methods. We disrupted the *csnA* gene of *S. lividans* TK24 applying the method involving a negative selection step on 5-fluorocytosine (Dubeau *et al.*, 2009), obtaining *S. lividans* TK24 Δ*csn*A::*neo*A. This mutant was devoid of any detectable chitosanase activity (not shown).

First, we determined the minimal inhibitory concentrations (MICs) of chitosan (Mn ≈ 10 kDa) on minimal agar medium for the wild type strain *S. lividans* TK24 and for *S. lividans* TK24 Δ*csn*A::*neo*A strain. The respective MICs were 0.08 g/l for *S. lividans* TK24 Δ*csn*A::*neo*A and 0.2 g/l for *S. lividans* TK24 (Figure 7). Abolition of chitosanase CsnA expression in the mutant strain increased 2.5 times its susceptibility to the toxic effect of chitosan.

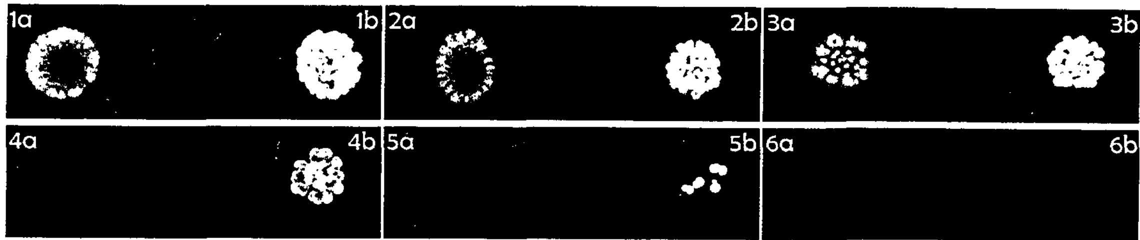

Figure 7: Determination of minimal inhibitory concentration of chitosan on agar plates. M14 agar medium was supplemented with 0 g/l (1), 0.05 g/l (2), 0.075 g/l (3), 0.08 g/l (4), 0.1 g/l (5) or 0.2 g/l (6) chitosan. 5 µl of spore suspension (diluted to $2x10^3$cfu ml^{-1}) of *S. lividans* TK24 Δ*csn*A::*neo*A (a) or *S. lividans* TK24 (b) were spotted on agar plates and incubated for 72h at 30°C.

We then studied the effect of the presence of chitosan in liquid culture medium on the viability of wild type (TK24) or CsnA-negative (TK24 Δ*csn*A::*neo*A) *S. lividans* strains. Those were also compared with *S. lividans* TK24 [pMPi-csnN106], a strain over-expressing a recombinant GH46 chitosanase originating from *Kitasatospora* sp. N106 (formerly known as *Nocardioides* sp. N106; Masson *et al.*, 1995). The uptake of xylose, the main carbon source added to this medium, was adopted as a measure of metabolic activity.

The rate of xylose comsumption in control cultures without chitosan was similar for all strains (Figure 8). At a permissive chitosan concentration of 0.1 g/l, the wild type and the CsnA-negative strains also behaved very similarly (46% of the initial xylose quantity was still present after 72 h of culture; panels A and B). But at a chitosan concentration of 0.2 g/l the xylose consumption was totally inhibited for the TK24 Δ*csn*A::*neo*A strain (panel B) while for the wild type strain inhibition was observed only at 0.3 g/l of chitosan (panel A). Finally, for the chitosanase overexpressing strain TK24 [pMPi-csnN106], an almost complete inhibition of xylose consumption was observed at the much higher chitosan concentration of 1.2 g/l (panel C).

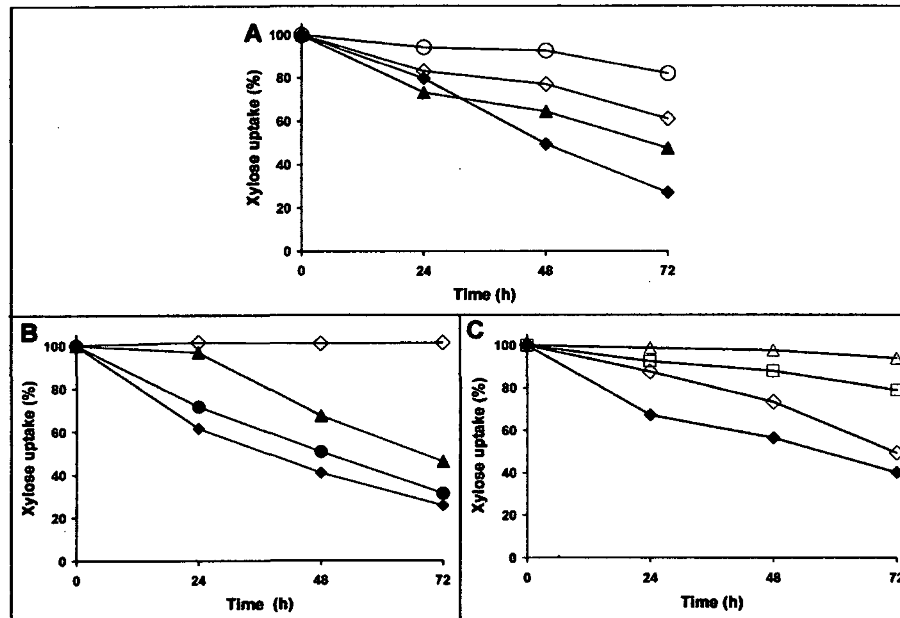

Figure 8: Effect of chitosan on xylose consumption by *S. lividans* strains. Minimal medium M14 was supplemented with 5 g/l xylose and 0 g/l (♦), 0.08 g/l (●), 0.1 g/l (▲), 0.2 g/l (◊), 0.3 g/l (○), 0.4 g/l (□) or 1.2 g/l (Δ) of soluble chitosan (3 kDa) and inoculated with *S. lividans* TK24 (A), *S. lividans* TK24 Δ*csn*A::*neo*A (B) and *S. lividans* TK24 [pMPi-csnN106] (C) strains. Xylose up-take was assayed by determining the residual level of reducing sugars (Lever *et al.*, 1972). Experiments were done in triplicate; one typical result is presented.

To verify how chitosan affected the growth of the respective strains in the tested conditions, 5 ml samples of cultures were collected periodically for dry mycelial weight determination. Growth data (not shown) were entirely consistent with the xylose uptake measurements. The culture supernatants from these cultures were also tested for chitosanase activity. No chitosanase activity was detected in the supernatants of the TK24 Δ*csn*A::*neo*A cultures, while TK24 and the TK24 [pMPi-csnN106] strains produced approximately of 23 mU/ml and 461 mU/ml of chitosanase, respectively.

DISCUSSION

In this study we determined the biochemical properties of the chitosanase CsnA from *S. coelicolor* A3(2). Our studies revealed that CsnA is, in many aspects, very similar to the chitosanase CsnN174 from *Streptomyces* sp. N174. However, several differences were

observed regarding pH dependence of various other parameters. CsnA enzymatic activity was uniform between pH 3.8 and pH 5.5 while that of CsnN174 showed a peak-shaped pH/activity profile. Also, spectrofluorimetric analysis showed that the intrinsic CsnA thermostability is pH dependent. Furthermore, the effect of chitosan (a polar polysaccharide) on thermostability was much more pronounced for CsnA at both pHs studied.

Chitosanase CsnA is secreted *via* the Tat pathway. Among the fully sequenced bacterial genomes, the amino acid sequence showing the greatest similarity to CsnA is that of chitosanase SAV2015 from *S. avermitilis* MA-4680. This protein could also be Tat-dependent, as revealed by a search using the TATFIND algorithm (Rose *et al.*, 2002).

Moreover, redirection of the CsnA secretion through the Sec-dependent pathway by fusing the *csn*A catalytic module encoding sequence with the signal peptide sequence of xylanase A (Li *et al.*, 2005), or that of cellulase A (this study), led to an important decrease in the secretion level of CsnA. The relatively poor efficiency of CsnA secretion through the SEC system may be due to inefficient folding of the protein in the extracytoplasmic compartment that could result in a high proportion of incorrectly folded and/or partially unfolded CsnA molecules.

Previous studies (Neugebauer *et al.*, 1991; Masson *et al.*, 1995) involving *S. lividans* concluded that this strain has no chitosanase activity. Here we used an improved medium for chitosanase production and detection and a more sensitive method for chitosanase assay (detection limit of 1mU/ml) with sRBB-C as substrate. *S. lividans* turned out to be chitosanase-positive but with very low levels of chitosanase secretion.

Analysis of the primary structure of CsnA from *S. coelicolor* and *S. lividans* revealed that these proteins have a very high degree of identity. Only three amino acids were differentiating the two chitosanases. The amino acid R37, corresponding to a histidine in CsnA from *S. coelicolor*, is localized in the signal peptide sequence, and it seemed not to affect chitosanase secretion in *S. lividans*. An amino acid sequence alignment of GH46 chitosanases

(shown in the Annexe I of this thesis) revealed a high variability of the residues occupying the positions Q110 and E241 (corresponding to E110 and Q241 in *S. coelicolor*). Thus, the CsnA from *S. lividans* may be considered as a neutral double mutant (E110Q + Q241E) of the CsnA from *S. coelicolor*.

The molecular weight of native chitosan is $0.1 - 4 \times 10^6$ Da, depending on its source (Wu *et al.*, 1976). Thus, chitosan must be hydrolyzed into smaller molecules before its assimilation by bacteria. Chitosanases may be considered as metabolic enzymes: as a result of endo-hydrolysis, the polymerization degree of chitosan is gradually diminished to dimers, trimers and tetramers. In function of the bacterial enzymatic baggage these chitooligomers are either transported for intracellular metabolism or further degraded to monomers. Thereby, some bacteria are secreting exo-chitosanases (Coté *et al.*, 2006), enzymes responsible for the degradation of the chitosan oligomers to monomers of glucosamine and *N*-acetylglucosamine. In some bacteria such as *E. coli, B. subtilis, S. aureus* and *Vibrio furnissii*, *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN) are takenup by the phosphotransferase system, which is responsible for their phosphorylation as well as their entrance in the cell. Then, *N*-acetylglucosamine is converted by a deacetylase to give glucosamine 6-phosphate. Glucosamine 6-phosphate is further converted by a specific deaminase to fructose 6-phosphate which will be metabolized through the glycolysis pathway (Riemann and Azam, 2002). On the other hand, some bacteria are able to transport and metabolize short chitooligomers, but the mechanism is unknown. The phosphotransferase system is also present in bacteria from the streptomycetes group such as *Streptomyces olivaceoviridis* and *S. coelicolor* where it was shown to be involved in the metabolism of GlcNAc and GlcNAc – GlcNAc, a result of chitin hydrolysis (Wang *et al.*, 2002; Rigali *et al.*, 2006). A newly identified ABC transporter NgcE (Xiao *et al.*, 2002) is also involved in chitin oligomers metabolism in these species. It is possible that similar transporters are involved in the transport and the metabolism of chitosan oligomers in *Streptomyces*. Moreover, studies on the metabolism of chitin in *Vibrio cholerae* revealed a new pathway involved in hydrolysis of this polymer. Chitin oligosaccharides $(GlcNAc)_n$, resulting from chitin hydrolysis by extracellular chitinases enter the periplasmic space via specific porins. Once in the periplasmic space, $(GlcNAc)_n$ oligomers are hydrolyzed

by two unique enzymes, a chitodextrinase and a β-*N*-acetylglucosaminidase (Park *et al.*, 2002). The hydrolysis products are further transported and phosphorylated by a phosphotransferase system similar to that from *E. coli* and *Vibrio furnissii.*

In the last few years, chitosan and its derivatives have received special attention as natural antimicrobial agents. Chitosan has a wide inhibition spectrum, from Gram-negative and Gram-positive bacteria, to yeast and moulds (Jeon *et al.*, 2001; Raafat *et al.*, 2008; Zakrzewska *et al.*, 2007; Rhoades and Roller, 2000). Recently, a new protective role against the antimicrobial effect of the chitosan has been suggested for chitosanases. Lacombe-Harvey *et al.* (2009) observed that expression of a heterologous chitosanase in *E. coli* conferred to the bacterial host a partial protection against the antimicrobial activity of chitosan. Further studies made in our laboratory showed that for *E. coli* strains, the protective effect was confined to the host strain and not extended to neighbouring cells, as *E. coli* does not secrete the chitosanase into the medium (Ghinet *et al.*, 2009). In contrast, a recombinant chitosanase secreted by a streptomycete strain conferred a protection for the neighbouring *E. coli* cells.

E. coli is naturally devoid of chitosanase activity. In this context, we were interested to see if the same role could be attributed to this enzyme, when produced by an organism naturally chitosanase-positive. Here we used a genetic approach to study the protective role of chitosanase against the bactericidal effect of chitosan. As shown by the *in vivo* experiments, the chitosanase-negative *S. lividans* TK24 Δ*csn*A::*neo*A strain was more sensitive to chitosan compared to wild type strain. At concentration as low as 0.08 g/l chitosan (10 kDa), the growth *S. lividans* TK24 Δ*csn*A::*neo*A on agar plates was totally inhibited compared to a concentration of 0.2 g/l of chitosan necessary to inhibit the wild type strain.

Interestingly, the *csnA* gene was not found to be essential for growth on minimal medium with chitosan as sole carbon source. Growth of the CsnA-negative mutant at permissive chitosan concentrations (such as 0.05g/l) was equivalent to that of the wild type strain. This may be explained by the presence in the *S. lividans* genome of a battery of genes encoding for chitinase activity (Neugebauer *et al.*, 1991; Miyashita *et al.*, 1993, Saito *et al.*, 1999; Fujii *et*

al., 2005). It is well documented, that many chitinases recognize various glycosidic linkage in the chitosan chain (Heggset *et al.*, 2009). In this rich genetic context, the protective role of the chitosanase CsnA against the antimicrobial effect of chitosan was the only phenotypic effect clearly visible when comparing the wild type and mutant strain, even if the wild type strain was found to produce very low amounts of chitosanase (in the mU/ml range).

Several authors have suggested that chitosan interferes with negatively charged components of the bacterial cell wall, which prevents nutrients from entering the cell (Zheng *et al.*, 2003). This corroborates our observations on xylose consumption. As shown by our *in vivo* studies, the xylose consumption by the chitosanase-negative mutant of *S. lividans* was inhibited at much lower chitosan concentrations compared with the wild-type strain and, especially with the chitosanase over-expressing strain. The latter was able to consume xylose at a chitosan concentration 6 times higher that the chitosanase-negative mutant. All these data sustain the concept that one of the phenotypic effects of chitosanase secretion is to protect the cells against the antimicrobial effect of chitosan

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CHAPTER IV

4.0 Cell wall localisation of the exo-chitosanase CsxA from *Amycolatopsis orientalis*

Amycolatopsis orientalis subsp *orientalis*, a Gram-positive bacterium belonging to the actinomycete group, has been studied for its chitosanolytic activity. Four enzymes involved in chitosan hydrolysis have been already identified from this soil organism: a chitinase, an endo-chitosanase (Csn), an exo-chitosanase (CsxA) and a β-N-acetylhexosaminidase.

The exo-chitosanase CsxA was intensively studied in our laboratory in terms of protein structure, catalytic features and enzymatic mechanism. CsxA participates in the final steps of chitosan degradation by hydrolyzing one glucosamine residue at a time, from the non-reducing end of the chitosan. CsxA belongs to the glycoside hydrolase family 2 (GH2). Lately, our attention was drawn by the modular structure of this enzyme. CsxA has four well defined domains: a sugar binding domain, an immunoglobulin-like beta-sandwich domain, a TIM barrel domain and a carbohydrate-binding module family 35 (CBM35). The latest module is a unique feature among GH2 members. The objective of this study was to understand the role that this module may play in the functionality of CsxA.

Carbohydrate-binding modules are non-catalytic polysaccharide-recognizing modules of glycoside hydrolases known to initiate the binding of those enzymes to their substrates. In a recent study, it has been shown that CBM35 does not bind to chitosan, the natural substrate for CsxA, but rather binds to glucuronic acid, a component of the Gram-positive bacterial cell wall. Therefore, we decided to analyze the cellular localization of the CsxA-CBM35 in *A.*

orientalis cells by epifluorescence and confocal microscopy in order to demystify the role of this unique association.

In this chapter, we present the epifluorescence and confocal microscopy results obtained for *A. orientalis* cells grown in presence of chitosan. Our results suggest that the CBM35 appended to the exo-chitosanase may function as a bacterial cell wall attachment module and could have as result a protective action against the antimicrobial effect of chitosan.

The work presented in this chapter was done under the supervision of Dr. R. Brzezinski. I purified by chromatography the CsxA protein and prepared it for hen inoculation. Dr. Brian Talbot realized the hen inoculation steps. I purified and characterized the anti-CsxA antibodies from hen eggs and realized their validation. The anti-CBM35 antibodies were provided by Dr. Boraston from U. of Victoria. I optimized the conditions for CsxA expression in *A. orientalis* and *S. lividans* TK24, and prepared the slides for microscopy. Mr. Gilles Grondin performed the microscopy work. Together with Dr R. Brzezinski, I analyzed the microscopy images and discussed the results presented in this chapter.

Evidence of chitosanase localization in the cell wall of the actinomycete *Amycolatopsis orientalis.*

4.1 Evidence of chitosanase localization in the cell wall of the actinomycete *Amycolatopsis orientalis.*

4.1.1 INTRODUCTION

Actinomycetes are Gram-positive bacteria known to be responsible for the singular odour emanating from the soil after rain. They play an important role in the decomposition of organic materials, such as cellulose and chitin and, thereby, they play a vital part in organic matter turnover and the carbon cycle.

The cell wall of Gram-positive bacteria accommodates a wide variety of molecules most of them being critical to the viability of the cell. Over their evolution, bacteria developed a number of unique mechanisms by which they can immobilize proteins on their surface. These mechanisms include either covalent attachment of proteins to peptidoglycan or the noncovalent binding of proteins to either the peptidoglycan or to secondary wall polymers such as teichoic and teichuronic acids.

Several examples of metabolic enzymes attached to the bacterial surface (Sahlstrøm *et al.*, 1993, Igarashi *et al.*, 2004; Goh *et al.*, 2006) are found in literature. The function of these enzymes is to break down large, nontransportable nutrient polymers into smaller subunits that can be subsequently transported into the cell by a permease system.

Cell wall localisation of the exo-chitosanase CsxA from *Amycolatopsis orientalis*

Amycolatopsis orientalis subsp *orientalis,* a Gram-positive soil bacterium, belonging to the

actinomycete group, has adapted to the presence of chitosan in its environment by expressing four enzymes involved in chitosan hydrolysis: a chitinase (belonging to the GH19 family), an endo-chitosanase (Csn), an exo-chitosanase (CsxA) (Kawase *et al.*, 2004; Sakai *et al.*, 1991, Côté *et al.*, 2006) and a β-N-acetylhexosaminidase (Nanjo *et al.*, 1990).

The exo-chitosanase (also known as exo-β-D-glucosaminidase) CsxA belongs to the glycoside hydrolase family 2 (GH2) and has a modular structure (Côté *et al.*, 2006). The sugar-binding domain, the immunoglobulin-like β-sandwich domain and the TIM barrel domain are characteristic for exo-β-D-glucosaminidases (GlcNases) belonging to GH2. However, the last domain of the CsxA, namely CBM35 (carbohydrate-binding module family 35), is unique among enzymes belonging to the GH2 family.

CBMs are non-catalytic polysaccharide-recognizing modules of glycoside hydrolases, known to initiate the binding of the enzyme to a high molecular form of the substrate; often in crystalline or colloidal form. The CBMs are divided into 59 families based on the amino acid similarity (http://www.cazy.org/fam/acc_CBM.html), and they display a high variability in ligand specificity. CBMs recognize crystalline and non-crystalline cellulose, chitin, xylan, mannan, galactan and starch (Boraston *et al.*, 2004).

Usually, CBMs are appended to glycoside hydrolases involved in the degradation of plant cell walls or solubilisation of storage polysaccharides such as starch and glycogen (Sigurskjold *et al.*, 1994, Boraston *et al.*, 2004). Those processes are very important for the maintenance of the terrestrial life due to their contribution to the diffusion into the soil of essential nutrients for all microbial ecosystems.

CBMs have three important functions in polysaccharide hydrolysis. Through their sugar-binding activity, the CBMs maintain the enzyme in proximity to the substrate. Thus, the increase in enzyme concentration at the surface of the substrate contributes to a more rapid degradation of the insoluble polysaccharide (Bolam *et al.*, 1998). CBMs have also a targeting function. Many interesting studies revealed the capacity of CBMs to recognise with high specificity, the reducing ends of polysaccharides, thus, targeting the enzymes to the damaged regions of the plant cell walls (Boraston *et al.*, 2001). More importantly, they help the enzyme to recognize different regions of the same polysaccharide and, by this; they influence the ability of the enzyme to hydrolyse the substrate (Boraston *et al.*, 2003). Furthermore, it was shown that CBMs can even initiate a non-catalytic disruption of the targeted substrate, thus, enhancing the degradative capacity of the catalytic module (Gao *et al.*, 2001).

As a general rule, the ligand specificity of bacterial CBMs reflects the substrate specificity of the carrier catalytic module. As shown by early studies (Nanjo *et al*,. 1990; Côté *et al.*, 2006) the exo-chitosanase CsxA recognizes the chitosan as its substrate. However, the carbohydrate-binding module (CBM35) appended to the catalytic module of CsxA has no detectable affinity for chitosan (Montanier *et al.*, 2009). Rather, it binds *in vitro* to glucuronic acid (Montanier *et al.*, 2009), a normal constituent of the cell wall of several actinobacteria (Asano *et al.*, 1990).

These observations have raised new questions concerning the role of the CMB35-CsxA association. We hypothesized that the CBM35 could anchor the CsxA enzyme to the cell wall through an interaction with the exposed surface polysaccharides. By increasing the effective enzyme concentration at the cell surface, the CBM35 could play a protective role against the antimicrobial effect of chitosan.

Here we analysed the CBM35-CsxA localization in *Amycolatopsis orientalis* cultures and we confirmed CBM35 ligand specificity by epifluorescence microscopy and confocal microscopy.

4.2 Material and Methods

4.2.1 Bacterial strains, plasmids and media.

Amycolatopsis orientalis subs. *orientalis* IFO12806 (ATCC 19795) and was obtained from the American Type Culture Collection, Manassas, VA, U.S.A. *Streptomyces lividans* TK24 (Kieser *et al.*, 2000) was used as host for the recombinant plasmids. The pFD666 shuttle vector was used for the expression of exo-chitosanase CsxA (Côté *et al.*, 2006) from *A. orientalis.*

4.2.2 Exo-chitosanase CsxA production and purification.

For CsxA production, spores of *S. lividans* TK24 (pFD666csxA) were inoculated in YME (yeast/malt extract medium) supplemented with 50 µg/ml of kanamycin and the culture was grown at 30°C with rotary shaking (240 rpm) for 72h. The mycelium was recovered by low speed centrifugation and then inoculated in M14 minimal medium (Pagé *et al.*, 1996) supplemented with 0.2% D-glucosamine and 0.8% Sigma chitosan (finely ground) as carbon source. After 72h of culture, the CsxA was purified as described previously (Côté *et al.*, 2006). The purity of the enzyme preparations was evaluated by 0.6% SDS-PAGE (Laemmli, 1970). Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard.

4.2.3 Polyclonal anti-CsxA antibodies production and purification.

We produced antibodies against the catalytic module of CsxA (devoid of CBM35) by injecting

the pure protein in hen (protocol BT01 2006/10/12 version 1). Animal manipulation steps were performed by Dr Brian G. Talbot. One hen was used in this experiment. Prior to immunization an egg was collected and served as control for the antibodies validation experiment. The first immunization dose consisted of 200 μg CsxA in 500 μl phosphate-buffered saline (PBS: 137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4) and 500 μl Titer Max Gold adjuvant (Sigma-Aldrich; St-Louis, MO, USA). This mixture was distributed into four sites of the pectoral muscle of the bird. Three weeks later, the hen was again immunized by the injection of 100 μg CsxA in 500 μl PBS and 500 μl Titer Max Gold adjuvant. Eggs were collected daily, beginning 5 days after the first injection, and stored at 4°C until analysis.

The anti-CsxA antibodies were purified from the eggs as described by Tini *et al.* (2002) with the following modifications. The yolk of the eggs was separated from the egg white, then, washed with distilled water to remove as much albumen as possible, and rolled on paper towels to remove adhering egg white. The yolks were pooled into 50 ml polypropylene tubes (Starstedt) and mixed with 0.02% sodium azide and 15 ml of 100 mM sodium phosphate buffer pH 7.6. Then, 20 ml of chloroform were added and everything was mixed well until a semi solid phase was obtained. Following 30 min. centrifugation at 1200xg, the supernatants were pooled, mixed with PEG 6000 (Polyethylene glycol; Sigma-Aldrich; St-Louis, MO, USA) to a final concentration of 12% (w/v), and then centrifuged at 13000 rpm for 10 min. The pellet containing the anti-CsxA antibodies was suspended in 0.2 ml of 100 mM sodium phosphate buffer pH 7.6 containing 0.02% sodium azide. The antibodies were conserved at -80°C. Purified antibodies concentration was determined by ultraviolet absorbance (280 nm) using a molar extinction coefficient of 14 M^{-1} cm^{-1}.

4.2.4 Anti-CsxA and anti-CBM35 antibodies validation by Western blotting

For the antibodies validation, 10 μl samples of supernatants collected after 24 h, 48 h, 72 h,

96 h and 120 h from a liquid culture of *S. lividans* TK24 pFD666csxA strain in M14 medium (with mannitol as carbon source) were subjected to 0.6% SDS-PAGE in 25 mM Tris/ 250 mM glycine/ 0.1% SDS buffer. After migration, the proteins were transferred to a PVDF membrane (Millipore, Billerica, MA, U.S.A.), using TBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20% methanol) (Burnette, 1981). The protein transfer was carried out at 100 Volts for 60 min. with the Mini Trans-Blot cell (Bio-Rad Laboratories). Then, the non specific binding sites were blocked by immersing the membranes in 5% non-fat dried milk, 0.1% (v/v) Tween 20 in TBS buffer for 16h at 4°C on a shaker. The primary chicken anti-CsxA antibodies (this study) or rabbit anti-CBM35 antibodies (kindly provided by Dr. Alisdair Boraston from the University of Victoria) were diluted to 1/10000 in TBS-T (TBS with 1% Tween 20) buffer containing 5% non-fat dried milk, prior to incubation with the membranes for 1h at 4°C. Membranes were washed with TBS-T four times for 5 min. each and then incubated with a 1:10000 dilution of donkey anti-chicken (Gallus Immunothech INC. ON, Canada) or donkey anti-rabbit (GE Healthcare Bio-Sciences) horseradish peroxidise-conjugated antibodies for 1h and the signal was developed with the ECL detection kit (GE Healthcare Bio-Sciences) by exposure to a Hyperfilm-ECL film.

4.2.5 Immunofluorescence microscopy

A. orientalis was grown in YME medium with shaking (250 rpm) at 30°C for 44 hours. The mycelium was recovered by centrifugation (4000 rpm, 5 min., 4°C) and inoculated (1 ml of the mycelial pellet per 100 ml of medium) into the induction medium (Côté *et al.* 2006) supplemented with 0.2% v/v glucosamine and 0.8% w/v chitosan (6% N-acetylated; ISM Biopolymer Granby, Canada). After 2, 3 and 24 hours, mycelium was harvested by centrifugation (30 sec, 130000 rpm). In the control experiments, the induction medium was replaced with YME medium. For epifluorescence microscopy and confocal microscopy, the supernatant was discarded and the mycelium was fixed by heat on Superfrost / Plus slides (Fisher Scientific).

The Alexa Fluor 488 goat anti-rabbit IgG #A-11008, Alexa Fluor 555 goat anti-chicken IgG #A21437, Prolong Gold antifade reagent # P36934, Hoechst 33342 #H-3570 were obtained from Invitrogen. The rabbit anti-CBM35 antibodies were kindly provided by Dr Alisdair Boraston from the University of Victoria.

Following the fixation on Superfrost / Plus slides the bacteria were permeabilized with 50% methanol at –20°C for 5 min and in PBSP (PBS with 50 mM of glycine, 0.06% saponine, 0.06% Tween 20, 0.5% NP40 and 0.5% TritonX-100) for 10 min. Smears were rinsed in PBSD (PBS with 0.01 % saponine and 0.01% Tween-20) for 5 min. at room temperature and the non-specific binding sites were blocked with PBSB (PBSD with 2% normal goat serum, 2% BSA and 0.45 % of Fish Gelatine) for 30 min. Smears were then incubated for 120 min. at room temperature with primary chicken anti-CsxA antibodies, and with rabbit anti-CBM35 antibodies or with rabbit anti-endochitosanase (anti-Csn) antibodies at a dilution of 1:1200 in PBSB. After several washes in PBSD, primary antibodies were detected by incubation with goat anti-rabbit Alexa Fluor 488 IgG and goat anti-chicken Alexa Fluor 555 IgG at a dilution of 1:200 in PBSB for 90 min. at room temperature. After several washes in PBSD and in water, the DNA was stained for 10 min. with Hoechst 33342 at a dilution of 1:5000. After several washes in water, slides were mounted in Plolong Gold anti-fade reagent. Pictures were taken using an inverted Olympus IX 70 microscope with a Cool SNAP-Pro cf monochrome camera and Image-Pro Plus software or with an Olympus Fluoview FV 300 confocal system.

For CBM35 binding competition experiments, the *A. orientalis* mycelium, previously incubated for 3 h in the induction medium supplemented with 1% (v/v) glucosamine, was washed twice for 10 min. on ice, with Tris-HCl 50 mM pH 7.5 containing 100 mM glucose or 100 mM glucuronic acid followed by centrifugation (4000 rpm, 5 min., 4°C). Before heat fixation, the mycelium was washed only with Tris-HCl 50 mM pH 7.5 in order to avoid glucuronic acid crystal formation on the slides.

4.3 Results

4.3.1 Cell wall localisation of the exo-chitosanase CsxA from *Amycolatopsis orientalis.*

4.3.2 Production and purification of anti-CsxA antibodies.

In order to analyze the CBM35-CsxA localization in *A. orientalis* cultures, we had to produce antibodies against the catalytic module of CsxA (devoid of CBM35).

The CsxA gene encodes for a 1032-amino acid protein. The N-terminal mature part (devoid of the signal peptide region) of CsxA, composed of 860 amino acids will be referred as the catalytic module for the rest of the study. The C-terminal module (CBM35) is composed of 126 amino-acids and is separated by a 6 amino-acids region from the catalytic module. In previous studies (Coté *et al.*, 2006; Fukamizo *et al.*, 2006), it was thought that the CsxA, purified following a long period of culture, was the full-length protein (CsxA with the CBM35). As determined by crystallography (van Bueren *et al.*, 2009) and confirmed by Western blotting results (Figure 1), CsxA undergoes specific processing by the bacterium during and/or after the export into culture supernatant. As a result, loss of the CBM35 module is observed. Thus, the CsxA protein obtained following purification represented only the catalytic module.

The catalytic module of the CsxA was purified from a culture of recombinant *S. lividans* pFD666CsxA as described previously (Côté *et al.*, 2006). Once purity of the protein was confirmed by 6% SDS-PAGE, CsxA was used for hen immunization (twice, at a three weeks

interval). The chicken anti-CsxA antibodies were purified from the eggs using an improved protocol (Tini *et al.*, 2002).

Before using the chicken anti-CsxA and the rabbit anti-CBM35 antibodies for the microscopy experiments, we verified their specificity by Western blotting. For that, 10 µl of supernatants collected after 24 h, 48 h, 72 h, 96 h and 120 h of culture of the *S. lividans* TK24 pFD666csxA strain in M14 medium (with mannitol as carbon source) were subjected to 0.6% SDS-PAGE, followed by Western blotting with chicken anti-CsxA and rabbit anti-CBM35 antibodies.

As shown in Figure 1, the Western blot confirmed the specificity of the antibodies. The anti-CsxA antibodies (raised against the catalytic module) recognize the two forms of the exo-chitosanase: the lower band (93 kDa) representing the catalytic module and the upper band (106kDa) representing the full length mature CsxA (with the CBM35 module attached) (panel A). The anti-CBM35 antibodies recognize only the full length exo-chitosanase (panel B).

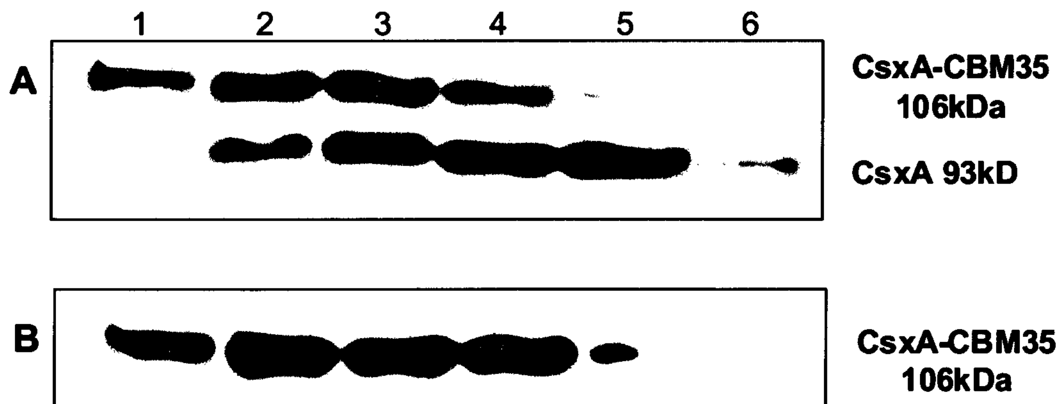

Figure 1: Validation of the chicken anti-CsxA and rabbit anti-CBM35 antibodies. 10 µl supernatant collected after 24h (1), 48h (2), 72h (3), 96h (4) and 120h (5) of culture of *S. lividans* TK24 pFD666csxA in M14 medium with mannitol as carbon source, and 50 ng of pure CsxA (6) were separated by 6% SDS-PAGE and transferred to PVDF membranes for Western blot analysis of anti-CsxA (A) and anti-CBM35 (B) antibodies specificity.

Furthermore, by Western blotting we confirmed the processing of the exo-chitosanase. As shown in panel A, in the supernatant of *S. lividans* TK24 pFD666csxA collected after 24 h of culture the full length form of CsxA is predominant, compared to supernatant collected after 120 h of culture in which the CsxA catalytic module form is predominant. The purified CsxA was used as control (panel A, row 6).

4.3.3 *In vivo* studies of the cell wall localization of the exo-chitosanase CsxA

In order to analyse the CsxA and CBM35 localization in *A. orientalis* cultures, the mycelium of *A. orientalis* was grown for two hours in YME medium (non inducing conditions) or M14 medium supplemented with 0.2% v/v glucosamine and 0.8% w/v chitosan (induction condition) prior to the epifluorescence microscopy analysis.

As may be observed the epifluorescence microscopy (Figure 2), CsxA (red) - CBM35 (green) expression was induced by the presence of chitosan in the culture medium (panels E and F), compared to the non inducing condition represented by the YME medium (panels A and B). Furthermore we could also observe that CBM35 and CsxA are co-localized (localization in a very tight proximity) (in orange) at the cell wall level when *A. orientalis* is grown in the presence of chitosan (panel H), compared to the non inducing conditions (panel D). The viability of the mycelium was confirmed by staining its DNA in blue with Hoechst 33342 (panels C and G).

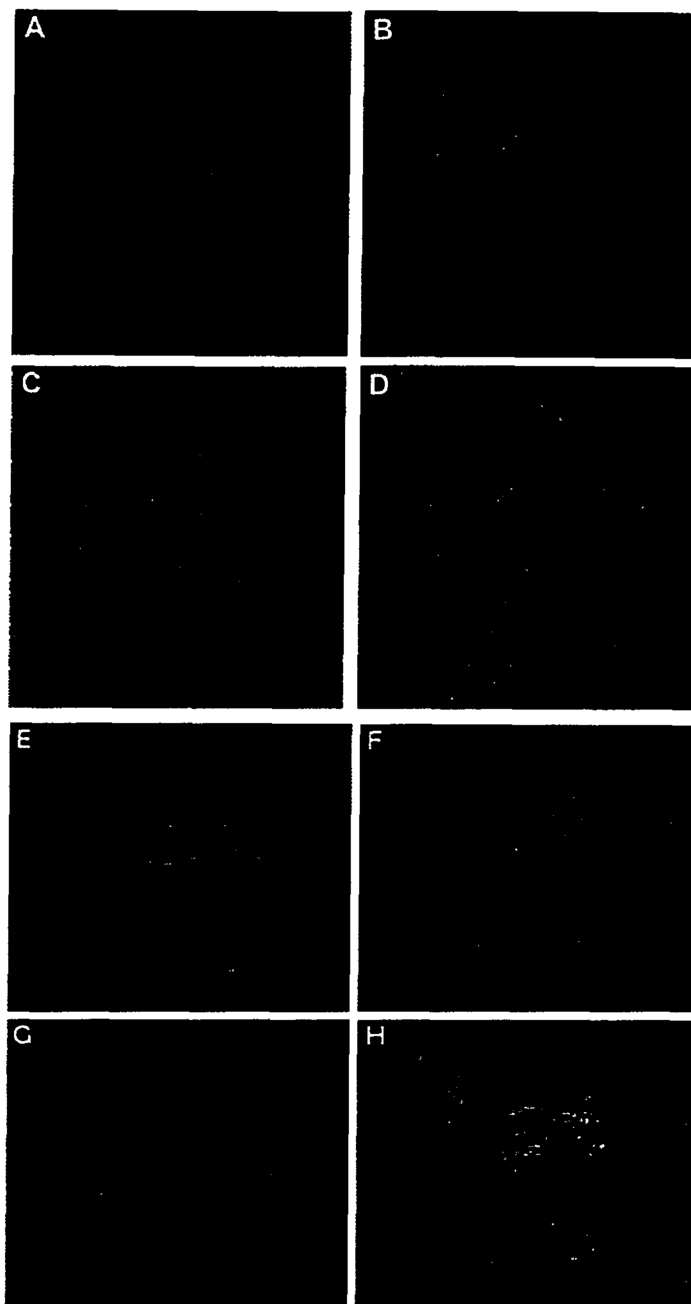

Figure 2: Epifluorescence microscopy analysis of the cell wall localization of CsxA and CBM35. *A. orientalis* was grown for two hours into YME medium (panels A to D) or into chitosan medium (panels E to H). The mycelium was harvested by centrifugation, fixed by heat on the microscope slides and then incubated with primary chicken anti-CsxA antibodies (panels A and E) and rabbit anti-CBM35 antibodies (panels B and F). Primary antibodies were detected by incubation with goat anti-chicken Alexa Fluor 555 IgG (red) and goat anti-rabbit Alexa Fluor 488 IgG (green). The DNA was stained with Hoechst 33342 (panels C and G). CsxA and CBM35 co-localization shown in orange (panels D and H) was obtained by superposing the immunofluorescence images showing the fluorescence of CsxA and CBM35.

In order to confirm the results obtained by epifluorescence microscopy we performed confocal microscopy analysis. For that, *A. orientalis* mycelium was grown for three hours into the M14 medium supplemented with 0.2% v/v glucosamine and 0.8% w/v chitosan. Then the mycelium was treated as described above, prior to confocal microscopy analysis.

These analyses (Figure 3) confirmed the co-localization of the CsxA and CBM35 (in orange) at the cell wall level of *A. orientalis*. Furthermore, the microscopy results are supported by earlier experiments showing that the CBM35 from CsxA binds to lyophilized *A. orientalis* cells (Montanier *et al.*, 2009).

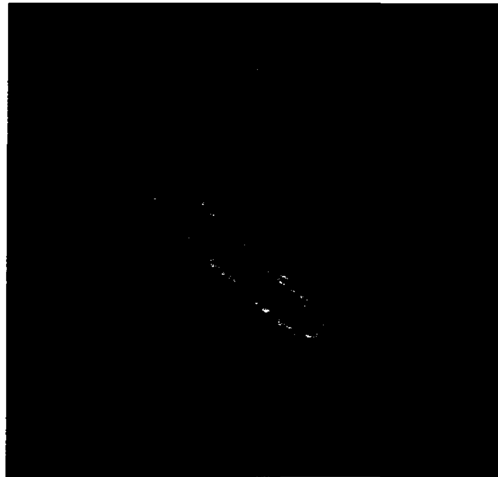

Figure 3: Confocal microscopy analysis of the cell wall localization of CsxA and CBM35.Image showing the CsxA (red) and CBM35 (green) co-localization (orange) at the *A. orientalis* cell wall level after 3 hours of induction in chitosan medium.

4.3.4 *In vivo* studies of the cellular localization of an anchored protein (CsxA) and a secreted protein (Csn).

We further analysed by confocal microscopy the difference in localization of an anchored protein (CsxA) and of a secreted protein (Csn) in *A. orientalis* cultures. For that, *A. orientalis* mycelium was grown for 24 hours in induction medium containing chitosan.

Figure 4: Confocal microscopy analysis of the cellular localization of a secreted protein (Csn; green) and an anchored protein (CsxA-CBM35; red) after 24 hours of induction. The heat-fixed mycelium was incubated on Superfrost / Plus slides with primary chicken anti-CsxA antibodies and rabbit anti-Csn antibodies. Primary antibodies were detected by incubation with goat anti-chicken Alexa Fluor 555 IgG (red) and goat anti-rabbit Alexa Fluor 488 IgG (green).

As shown in Figure 4, by confocal microscopy we could observe the difference between a classical secreted protein, the endo-chitosanase (Csn; green), and an anchored protein, the exo-chitosanase (CsxA; red). This figure shows that the chitosanase Csn, a protein known to be secreted into the extracellular medium (Sakai *et al.*, 1991) is present all around the bacteria, in a cloudy diffuse halo. Very few Csn molecules were observed at the cell wall level (these few molecules were probably just transiting through the wall). In contrast, CsxA was mostly found anchored to the cell wall. CsxA was also present in the "extracellular medium" probably in the processed form (without CBM35). This processing was observed in *S. lividans* (Figure 1) as well as in *A. orientalis* (data not shown). All those results are in accordance with the hypothesis anchorage of the CBM35 to the cell wall.

4.3.5 *In vivo* studies of the CBM35 ligand specificity.

I*n vitro* studies realized by Montanier and coworkers (Montanier *et al.*, 2009) led to the

identification of the CBM35's ligand: glucuronic acid. Glucuronic acid is known to be a component of glycosyl diacylglycerols which are glycolipids localized in the membrane of some Actinomycetes (Batrakov *et al.*, 1978, Chatterjee *et al.*, 1988). It is also a component of teichuronic acids present in the cell wall of several Gram-positive bacteria (Weidenmaier and Peschel, 2008).

In order to verify if CBM35 is anchored to the cell wall *via* interaction with the uronic acid sugars, we analysed by epifluorescence microscopy the difference in cell wall localization of CsxA and CBM35, between *A. orientalis* mycelium (prealably grown on chitosan) washed with glucose or washed with glucuronic acid. Interestingly, we observed a great difference between these two conditions. As expected, washing with glucose (control condition) did not affect the cell wall localization of CsxA and CBM35. Instead, washing with glucuronic acid drastically decreased the CsxA and CBM35 specific signals at the cell wall level. These results suggest that glucuronic acid acts as a stong eluent for the CsxA-binding component localized at the cell wall level.

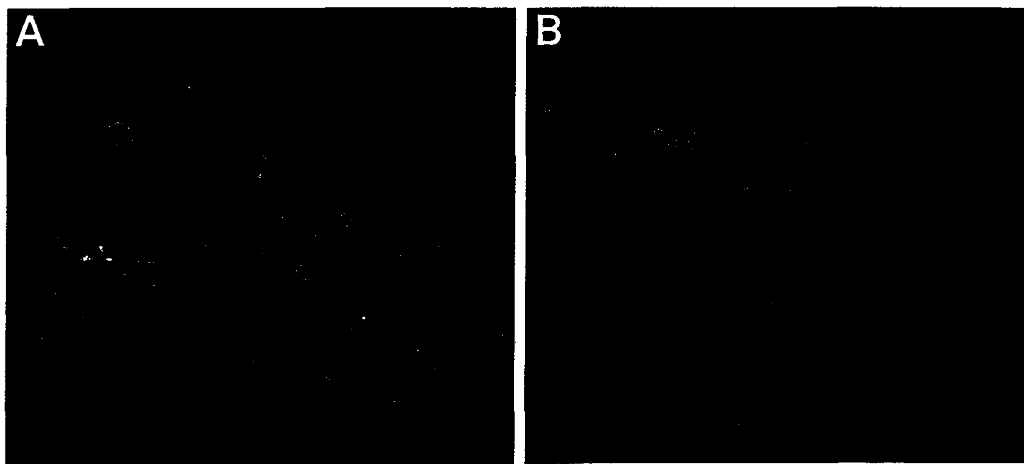

Figure 5: Epifluorescence microscopy analysis of the CBM35 ligand specificity. CsxA (red) and CBM35 (green) co-localization at the cell wall level. The bacterial DNA was stained in blue. *A. orientalis* was grown for three hours in glucosamine medium. Before heat fixation the

mycelium was washed twice for 10 minutes, on ice, with 100 mM glucose (A) or 100 mM glucuronic acid (B).

4.4 Discussion

The exo-chitosanase CsxA has an uncommon structural module among the exo-β-D-glucosaminidase members of the GH2 family, namely the CBM35 (carbohydrate-binding module belonging to the family 35). Unlike most of the carbohydrate binding modules, whose main function is to bind the glycohydrolase substrates in order to facilitate hydrolysis processes, CBM35 appended to the CsxA does not recognize chitosan as ligand.

The ability of this CBM35 to recognize glucuronic acid as substrate, a constituent of bacterial cell wall, suggested a new function as a bacterial cell wall attachment module.

By Western blotting analysis, we detected only traces of the full length protein form (103 kDa) in the supernatants of *A. orientalis* at early culture stages (2-3 h) after chitosan induction (data not shown). These observations indicated that CsxA is a secreted protein, that exists as a full length 103 kDa form when non-covalently attached to the cell wall of *A. orientalis* via a CBM-carbohydrate interaction and becomes released into the supernatant at later culture stages via C-terminal processing. The association with the cell wall is mediated through CBM35 module interaction with the glucuronic acid. Furthermore, CsxA does not display any other Gram-positive cell wall binding motifs such as transmembrane domains, sortase-mediated attachment motif (LPXTG) or choline-binding domains (CPB) (Montanier *et al.*, 2009).

As shown by the time course experiment, CsxA C-terminal processing was also observed in *S. lividans* cultures expressing the recombinant CsxA-CBM35 protein, but with this strain the processing was much slower compared to *A. orientalis*. This suggests the presence of a panoply of different proteases responsible for CsxA processing.

The analyses made by epifluorescence microscopy, showed that chitosan induces the expression of the CsxA-CBM35 in *A. orientalis* cultures. The co-localization of CsxA and CBM35 at the cell wall level observed by epifluorescence microscopy was confirmed by confocal microscopy analysis. Thus, the *in vivo* experiments, presented in this chapter, concerning the capacity of CMB35 to bind the glucuronic acid, are consistent with the *in vitro* experiments realized by Montanier and coworkers. The CBM35 appended to the exo-chitosanase has a unique capacity to function as a bacterial cell wall attachment module. The microscopy results presented in this chapter are the first evidence of bacterial chitosanase localization at the cell wall level.

There is a simple biological explanation for these results. CBM35 attachment to the bacterial cell wall favours an accumulation of the CsxA at the bacterial surface. The bacterial surface is in direct contact with the chitosan, known for its antimicrobial effect. The polycationic nature of the chitosan, brought by the positively charged NH_3^+ groups of the glucosamine monomers, is a major factor contributing to its antimicrobial activity. This polycationic structure can easily interact with the predominantly anionic components of the Gram-positive bacteria cell surface such as negatively charged cell wall polymers (teichoic or teichuronic acids and anionic polysaccharide) anchored to the peptydoglycans. The accumulation of CsxA at the cell wall will lead to a faster hydrolysis of the chitosan and contribute to cell survival.

Moreover, *A. orientalis* also secretes an endo-chitosanase (Csn) (Sakai *et al.*, 1991) in the presence of chitosan. The secreted Csn will be the first enzyme to interact with chitosan and to

start hydrolysis. It is well documented that the antimicrobial activity of the chitosan is directly influenced by its molecular weight. Chitosan and its derivatives with molecular masses in the 10–20 kDa range are the most active as bactericidal molecules (Liu *et al.*, 2001; Park *et al.*, 2004). In this context, chitosanase activity could transiently intensify the antimicrobial effect of chitosan unless chitosan it is rapidly hydrolyzed into short oligosaccharides. The hydrolysis products will be further degraded by the exo-chitosanase CsxA attached to the bacterial cell wall. The exo-chitosanase removes single glucosamine residues from the non-reducing terminal of chitooligosaccharide and in combination with the β-N-acetylhexosaminidase secreted by *A. orientalis* (Nanjo *et al.*, 1990), completes the chitosan hydrolysis. The monosaccharide products of this combined hydrolysis, devoid of antimicrobial activity, will then be easily transported into the bacterial cell for further metabolism.

In conclusion, our results suggest that *A. orientalis* found an original way to resist to the antimicrobial action of chitosan. By expressing two chitosanases with different mechanism of chitosan hydrolysis (endo-hydrolysis *vs* exo-hydrolysis), *A. orientalis* does not only survive in the presence of chitosan but is also able to use it efficiently as a carbon and nitrogen source.

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CONCLUSION

In recent years, chitosan, a biodegradable and nontoxic biopolymer for humans, has emerged as a new potential biomaterial for agriculture, food industry, pharmacology and medicine. Lately, this polysaccharide was intensively studied due to its antimicrobial activity against bacteria, fungi and viruses. Different potential commercial applications of chitosan benefiting from its antimicrobial activity such as food preservation, antimicrobial treated textiles and wound healing bandages, are under intense studies.

Many research groups evaluated the mechanism of antimicrobial activity of chitosan. They showed that the degrees of acetylation and polymerization are important factors for the bactericidal effect of chitosan. Therefore, chitosanases, enzymes responsible for chitosan depolymerization, indirectly influence the antimicrobial activity of chitosan. In an ecological context, it is thought that the presence of chitosanase producing strains and chitosan of fungal origin may have an influence on the growth of other bacteria lacking chitosanase activity, thereby, they may change the ecological balance.

The goal of this PhD project was to elucidate the phenotypic effects of chitosanases in the producing organisms. The project was structured into four parts.

The first part was dedicated to studies on 1) the effect of the molecular weight of chitosan on its antimicrobial activity and 2) the influence of chitosanase production on the survival of *E. coli* strains (naturally devoid of chitosanase activity) in the presence of chitosan. These studies showed that expression of an heterologous chitosanase in *E. coli*, increased the level of resistance to chitosan. When the heterologous chitosanase was inactive, no such protective

effect against the chitosan was observed. Moreover, we observed that secretion of an extracellular chitosanase by a *S. lividans* strain can protect neighboring chitosanase-negative bacteria against the antimicrobial effect of chitosan. The secreted chitosanase diffuses into the medium and hydrolyzes the polymer into oligosaccharides devoid of inhibitory properties. In *E. coli*, the chitosanase had an intracellular localization, thereby this protective effect on the neighboring chitosanase-negative bacteria was not observed. The experiments performed on solid medium are comparable in some aspects to soil conditions. Thus, we can assume that in soil, as well as under laboratory conditions, chitosanase production has two important roles in bacteria: a metabolic role by which chitosanases contribute to the utilization of the chitosan as carbon and nitrogen source and a protective role against the antimicrobial effect of chitosan. These studies brought new evidence for the protective effect that chitosanases exert on the expressing bacteria when found in the presence of chitosan.

Actinobacteria are considered to be one of the largest phyla among bacteria and represent Gram-positive bacteria with a high G+C content. Actinobacteria members have adapted to different life environments. They can be pathogenic for humans (e.g., *Corynebacterium, Mycobacterium, Nocardia*), soil inhabitants (*Streptomyces*), or gastrointestinal commensals (*Bifidobacterium*). Actinobacteria are well known as secondary metabolite producers therefore being of high pharmaceutical interest. In this context, actinobacteria have been subjected to intensive genetic studies.

The second part of this project was dedicated to the development of a new system for gene disruption and replacement in actinobacteria. Genetic manipulations such as gene disruption or replacement are realized with the purpose of determining the biological role of the gene of interest. In this study, the new system was used for three different genetic manipulations in *Streptomyces lividans* and all of them were successful. This is a simple, time-saving and also money-saving method. This represents the first application of the cytosine deaminase suicide

selection system in bacteria. The development of this new system represents an important contribution for future genetic manipulations in actinobacteria.

The third part of this PhD project was dedicated to the study of the biochemical properties and the phenotypical effect of the chitosanase from *Streptomyces coelicolor* A3(2). The CsnA is the first chitosanase secreted by the Tat-dependent pathway to be characterized. Hence, these studies improved our knowledge about the chitosanase secretion.

Amino acids sequence analysis of the secreted CsnA, revealed that its *N*-terminus occurred three residues later than the one predicted by SignalP 3.0 algorithm. Interestingly, in *Streptomyces lividans*, a tripeptidyl aminopeptidase (Tap) was found to be responsible for the removal of tripeptides from the *N*-terminus of secreted recombinant proteins (Krieger *et al.*, 1994). The tripeptide present in the *N*-terminus of CsnA (S-A-R) is different than the one recognized as substrate by Tap (G-P-L) (Butler *et al.*, 1995). Interestingly, by BlastP analysis we found that *S. lividans* has at least four other secreted proteases sharing between 37 and 51% of homology with Tap. Thereby we can hypothesize that one of those peptidases may be responsible for this post-secretory processing of CsnA.

Furthermore, by disrupting the chitosanase gene in *Streptomyces lividans* we obtained genetic evidence that support the results described for *E. coli* concerning the protective role of chitosanases against the bactericidal activity of chitosan.

The chitosanase negative *S. lividans* strain grew normally at low concentrations of chitosan compared to the wild type strain. However, further investigations are required in order to understand the effect of chitosanase disruption on the expression of other genes involved in the chitosan metabolism. Moreover, it will be interesting to analyze the expression of genes

related to the membrane stress in the wild type *S. lividans* strain and in the mutated strain grown in the presence of chitosan. It was also assumed that in fungi chitosanase may be involved in cell differentiation. The gene SCO7070, coding for a chitosanase member of the GH75 family, has a TTA leucine codon, identified as a possible target for *bldA* regulation. In this context SCO7070 is a very good candidate for further gene manipulation experiments. This may help us to discover other physiological roles that chitosanases may have in bacteria.

The fourth part of this PhD project studied the cellular localization of the exo-chitosanase CsxA from *Amycolatopsis orientalis* in an effort to understand the function of the carbohydrate binding module (CBM35) appended to this glycoside hydrolase. Microscopy analysis of the cellular localization of CsxA and CBM35 showed that CsxA was attached to the bacterial cell wall. The association with the cell wall is mediated through CBM35 module interaction with glucuronic acid. Glucuronic acid is a part of the teichuronic acid, a Gram-positive cell wall structural acidic polysaccharide. It was shown that the antimicrobial activity of chitosan is based on its capacity to bind the negatively charged components of the cell wall. All these observations may suggest that the positively charged NH_3^+ groups of glucosamine monomers from chitosan could interact with the carboxyl teichuronic acid groups from the *A. orientalis* cell wall. Interestingly, by crystallography, it was shown that the CBM35 forms non-covalent interactions with the carboxyl group of the glucuronic acid. Hence, CsxA-CBM35 and chitosan compete for the same substrate in order to exert their respective functions. Our results showed CBM35 functions as a bacterial cell wall attachment module for the exo-chitosanase CsxA. CsxA accumulation at the level of the cell wall offers a protection against the antimicrobial activity of chitosan first by hydrolyzing the chitosan oligomers to glucosamine monomers, devoid of any bactericidal activity, and second by blocking the access of chitosan to the negatively charged residues from the bacterial cell wall. CsxA is the first exo-chitosanase reported to posses a CBM. These studies lead to the identification of a new biological role for the carbohydrate binding module (CBM35). Moreover, the results obtained in this study present valuable evidence for the contribution of chitosanases to the resistance against chitosan.

Our recent studies have improved our knowledge concerning the physiological role of chitosanases in bacteria. Moreover, they show how well bacteria adapted to the presence of chitosan in the soil. Bacteria posses a very complex battery of metabolic enzymes specialized for chitosan hydrolysis. In response to chitosan toxicity, they adapted by producing efficient chitosanases for hydrolyzing this diversified natural polymer. The acquirement of CBM35 appended to CsxA represents also a remarkable evidence of bacterial adaptation to the presence of chitosan.

To date, more than 1040 bacterial genomes have been sequenced. These studies put in evidence the presence in bacterial genomes of genes with interesting biotechnological activities such as cellulase, galactosidase and xylanase. Interestingly, very few potential chitosanase genes were discovered following these genome sequencing projects. Most of the chitosanase activities were rather discovered through the screening of various natural environments for chitosanase producing bacteria. This may indicate that chitosanase activities are rare among bacteria, compared to other glycoside hydrolases activities such as cellulase and xylanase.

In nature, most of the chitosanase activities are found in bacteria and fungi. Among bacteria, chitosanase activities are mostly found in Gram-positive bacteria and only rarely in Gram-negative bacteria. Interestingly, the Gram-negative bacteria are less sensitive to the presence of chitosan compared to Gram-positive bacteria. The mechanism of the chitosan antimicrobial activity is based on its cationic character and involves bacterial cell lysis, breakdown of the cytoplasmic membrane barrier and the chelation by chitosan of the trace metal cations necessary for the microorganism's growth. The antimicrobial mode of action of chitosan is dependent upon the microorganism. In Gram-negative bacteria, the protective barrier is conferred by the outer membrane. The lipopolysaccharides (LPS) from the outer membrane mask the negative net charges by providing a partial hydrophobicity (lipoprotein A) and by minimizing the exposure of unsubstituted acidic groups. It has been shown that the number of

LPS molecules preset in the outer membrane has an important influence on the level of bacterial sensitivity to chitosan. In Gram-positive bacteria, in which the outer membrane is absent, the barrier to overpass is represented by the layer of peptidoglycan which is associated with the negatively charged molecules of teichoic and teichuronic acids, respectively. Thereby, the surface of Gram-positive bacteria is thought to be more negatively charged than the Gram-negative bacteria, which may explain the difference in their sensitivity to chitosan. On the other hand, the peptidoglycan is a remarkably dynamic molecule. In Streptomyces coelicolor, over 60 different genes encode for cell wall hydrolases responsible for peptydoglycan modelization during the cellular differentiation (Haiser *et al.*, 2009). We can hypothesize that chitosan may interfere with the cellular differentiation by binding the substrates of different cell wall hydrolases and rendering them less accessible for hydrolysis. This hypothesis is sustained by a slight difference observed in the aerial mycelium formation by the chitosanase negative mutant grown in the presence of chitosan. Moreover, Gram-negative bacteria can often use the small chitosan oligomers (<2 kDa) as nutrient source as their growth is sustained by dimers and trimers of chitosan, without having any chitosanase activity to explain this resistance to chitosan. In this context, some may say that Gram-negative bacteria don't have chitosanase activities because they don't need them, due to their natural increased resistance. Nevertheless, in the context in which chitosan is becoming more and more popular in areas such as agriculture (plant protection against pathogens) or medical treatment (chitosan as hypocholesterolemic agent), one question may be raised: how bacterial populations of the respective habitats (soil or human gut, respectively) will react to the increasing concentrations of this polymer? If our observations can be extrapolated to these complex habitats, the use of chitosan would result in changes in the bacterial populations, favouring chitosanase-producing species. The evaluation of this "side effect" of chitosan application will have to be included in future research. In this context it will be interesting to verify if bacteria from these habitats will acquire chitosanase genes by horizontal gene transfer and if chitosanase genes will be found as being a part of the mobile genetic elements in bacteria exposed to increased concentrations of chitosan, thereby behaving similarly to the antibiotic-resistance genes as a result of generalized use of antibiotics in therapy. Such an

ecological behaviour would provide a further argument to the phenotypic function of chitosanase as a resistance factor against the antimicrobial effect of chitosan.

ANNEXE I

Alignment of the amino acid sequences of the chitosanases members of the GH46 family. The alignement was obtained by the T-COFFEE (Version 7.71) structure guided alignement method (Notredame *et al.*, 2000). The peptide signal sequence and the N-terminal extensions of certain proteins were deleted. The arrow indicates the first amino acid of the mature part for most of the chitosanases, when this amino acid was determined.

```
STR_N174       -------------stqastavkagaglddphkkeiamelvssaenssld
NOC_N106       -----------p-ap-agatrlaavglddphkkdiamqlvssaenssld
AMY_CS02       ---------apa-apaaaavsvasvglddpakkeiamelvssaenssld
BAC_amMJ1      -------------t-lmmnntvfaaglnkd-qkrraeqltsifengmt-
BAC_CIRC       ---------dnfs-peslqflrnntgldgeq-wnnimklinkpeqddln
BAC_KFB        -------------t-lmmsetvfaaglnkd-qkrraeqlrricedgtt-
BAC_SUBT       -------------t-lmmsetvfaaglnkd-qkrraeqltsifengtt-
BUR_GLAD       ---------anfs-patlqflkdntgldgeq-wdnimklvnkpeqdsld
SAV2015        -------------a--nqsataapvglddpakkeiamklvssaenssld
SAV6191        ---------esp-kppsrqssasaqglnapakkelaqeivasaenstld
SCO0677        -------------pshaaaasaratglddpakkeiamqlvssaenssld
STR_LIV        -------------PSRAAAASARATGLDDPAKKEIAMQLVSSAENSSLD
PSE_A01        ---------------aasveaagtvdldapvqkdtamslvssfensstd
STR_AM         gyslyeveayg-agattpppssgtglddprkkevamklvssfenssld
STR_AMBO       -------------ppraaatparaaglddpakkeiamrlvssaenssld
STR_SCL        -------------a--paaahaapaglddpakkeiamqlvssaenssld
STR_CLAV       ---------YRA-APAAPAGRASGPGLDNPVKKDIAMRLVSSAENSSLD
CHR_VIOL       ---------anfp-patlkflkantgldgeq-wdnimklvnkpeqdsle
BAC_amMJ1_1    -------------t-lmmnntvfaaglnkd-qkrraeqltsifengmt-
BAC_amFZ       -------------t-lmmsgtvfaaglnkd-qkrraeqltsifengkt-
PAENIBAC       ---------snff-patlmplrdntgldgeq-wnnimmlvnkpeqddln
PAE_EHIM       ---------enfs-peslqflrdrtgldgeq-wnnimklinkpeqddln
MICROB         ---------------aasaetagtvdldapvqkdtamslvssfensstd
REN_SALM       ---------sslapaltavsaastgdlsapakkeiamqlvcsaenssld
BAC_DAU        -------------t-lmmsgtvfaaglnkd-qkrraeqltsifengkt-
CBEI_0949      ----------------SESSAAEAITANS-EQKAVDLQLTTVCENSKTK
CHV_CVK2       ---------dthi-psqveaplkklgfntt-nadtilslialpensttq
CHV_PBU        ---------dthi-psqveaplkklgfntt-nadtilslialpensttq
PLAV_0939      ----------------------------selqkeaakaivnifetgavr
CELL_JAP       ----------------------------tptqkktaeaivnifetgsvl
DARO_2852      -------------p-slpstr-rswkmltptqkqtaqsivnlfetgavl
DARO_2340      ----------------------------tptqkqtaqsivnlfetgavl
NPUN_R2009     -------------ppgtlnqlntltgldaq-qltnilgmingpeqansk

cons                                        :     *
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```
STR_N174        w-------kaqykyiedig--dgrg-ytggiigfcsgtg-----dmlel
NOC_N106        w-------ksqykyiedik--dgrg-ytagiigfcsgtg-----dmldl
AMY_CS02        w-------kaqykyiedig--dgrg-ytagiigfcsgtg-----dmlel
BAC_amMJ1       --------eiqygyvehlp--dgrg-ytcgragfttatg-----dalev
BAC_CIRC        w-------ikyygycedie--derg-ytiglfgattggsrdthpdgpdl
BAC_KFB         --------emrypyvarld--darp-stcgpagvttatr-----dgfev
BAC_SUBT        --------eiqygyverld--dgrg-ytcgragfttatg-----dalev
BUR_GLAD        w-------tkfygycedig--ddrg-ytmgifgattggpndggpdgpal
SAV2015         w-------kaqykyiedig--dgrg-ytagiigfcsgtg-----dmldl
SAV6191         w-------rsaygyvedig--dgrg-ytagvigfctgth-----dlltl
SCO0677         w-------kaqyryiedig--dgrg-ytagiigfcsgtg-----dmldl
STR_LIV         W-------KAQYRYIEDIG--DGRG-YTAGIIGFCSGTG-----DMLDL
PSE_A01         w-------qaqygyledia--dgrg-ytggligftsgtg-----dmlel
STR_AM          w-------raqfayiedid--dgrg-ytagiigfcsgtg-----dmlel
STR_AMBO        w-------kaqygyiedir--dgrg-ytagiigfcsgtg-----dmldl
STR_SCL         w-------kaqygyiedig--dgrg-ytggiigfcsgth-----dmlel
STR_CLAV        W-------EAQYSYIEDIG--DGRG-YTAGVAGFCSGTG-----DMLDL
CHR_VIOL        w-------tkfygycenig--dkrg-ytigifgattggpndegpdgptl
BAC_amMJ1_1     --------eiqygyvehlp--dgrg-ytcgragfttatg-----dalev
BAC_amFZ        --------eiqygyveald--dgrg-ytcgragfttatg-----dalev
PAENIBAC        w-------idfygycedid--ddrg-ytivlfgattggsndtgpdgpdl
PAE_EHIM        w-------ikyygycedin--derg-ysigifgattggprdthpdgpel
MICROB          w-------qaqygyledia--dgrg-ytggligftsgtg-----dmlel
REN_SALM        w-------kaqygyiedid--ddrg-ytggiigftsgtg-----dmlel
BAC_DAU         --------eiqygyveald--dgrg-ytcgragfttatg-----dalev
CBEI_0949       ---------FVYNIAEKLGSFDGRG-ITFGCIGFTTGTY-----DGNIL
CHV_CVK2        w-------wknynyasclk--dgrg-wtvtiygacsgtg-----dllmv
CHV_PBU         w-------wknynyasclk--dgrg-wtvtiygacsgtg-----dllmv
PLAV_0939       ---------gdyarvtlla--gdsghltygraqttlgsg-----nlhll
CELL_JAP        ---------gdysnvtlit--gdtghltfgrsqttlgsg-----nlgkl
DARO_2852       ---------ghygnvtvip--gdtghltfgrsqttlcsg-----nlldl
DARO_2340       ---------ghygnvtvip--gdtghltfgrsqttlcsg-----nlldl
NPUN_R2009      wwqtvdeeiiygyaedie--dgrg-vtigiygattgkgyn---dadvi

cons                      :     :    .     :                :   :
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E110

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STR_N174      vqhytdlepg---nilakylpalkkvngsashs-g-------lgtpft
NOC_N106      vadytdlkpg---nilakylpalrkvngtesha-g-------lasafe
AMY_CS02      vqhytdlkpg---nvlakylpalkkvngtdshs-g-------lgsafv
BAC_amMJ1     vevytkavpn---nklkkylpelrrlakeesdd-is------nlkgfd
BAC_CIRC      fkaydaakgas--np--sadgalkrlgingkmk-gsileikdsekvfc
BAC_KFB       vpvykqavan---kklpnylaglrrlekeasdd-ts------klkgpa
BAC_SUBT      vevytkavpn---nklkkylpelrrlakeesdd-ts------nlkgfa
BUR_GLAD      fkaydaasgas--np--svqgglarigahgsmq-gsilkitdsekvfc
SAV2015       velytqrkpg---nvlatylpalrnvnggdshq-g-------ldpgfp
SAV6191       verytkdhpg---nglarflpalravdgtdsha-g-------lgraft
SCO0677       velygerspg---nvlapylpalrrvdgsdshe-g-------ldpgfp
STR_LIV       VELYGQRSPG---NVLAPYLPALRRVDGSDSHE-G-------LDPGFP
PSE_A01       vraysasspg---npleqyipaleavngtdsha-g-------lgqgfe
STR_AM        verytakkpg---nplarylgalravdgtdshe-g-------ldpgft
STR_AMBO      velytarspg---nvlarhlpalrevdgsdshe-g-------ldpgyp
STR_SCL       velytqrkpg---nvlakylpalrevdgsdshe-g-------ldpgft
STR_CLAV      VELYTERKPG---NALARYLPALRKVDGSDSHR-G-------LDPNYP
CHR_VIOL      fkefdassgas--np--saqglaraqvhgsmq-gkilkisdsakvfc
BAC_amMJ1_1   vevytkavpn---nklkkylpelrrlakeesdd-is------nlkgfd
BAC_amFZ      vevytkavpn---nklkkylpelrrlakdesdd-is------nlkgfa
PAENIBAC      fkaydaakgas--np--svkgalarigvkgsmk-gkileineseesfc
PAE_EHIM      fkaydaakgag--np--svegalkrlgingkmk-gsileikdsekvfc
MICROB        vrahsasspg---npleqyipaleavngtgsha-g-------lgqgfe
REN_SALM      vqnyantkpdn--nvlkpflpvlrkvngtkshe-g-------lgqkyv
BAC_DAU       vevytkavpn---nklkkylpelrrlakdesdd-is------nlkgfa
CBEI_0949     IHYYTKLNPN---NNLAKYIPALDRIDKEEHDEDGKNDDIKGL-DNFI
CHV_CVK2      leslqkinpn---hplvkfipamrktkgddi-r-g-------l-enlg
CHV_PBU       leslqkinpn---hplvkfipamrktkgddi-r-g-------l-enlg
PLAV_0939     ikaydeapgaayaracepylprladidlrld------------sdwtfr
CELL_JAP      lqlycanpgarfrgqltpflarfaardfsld-----------heehlk
DARO_2852     lqrycgndgarfgdklsawlarfeavdlsld-----------ddlhlh
DARO_2340     lqrycgndgarfgdklsawlarfeavdlsld-----------ddlhlh
NPUN_R2009    wknygqdysn---lpv---------------------------deii
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cons

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NOC_N106      kdwataa-kdsvfqqaqnderdrsyfnpavnqaka-s---------lr
AMY_CS02      ndwrtaa-kdtvfqraqnderdrvyfnpavkqakaer---------lr
BAC_amMJ1     sawrslg-kdkdfraaqdtvndrlyyqpamkqsdnig---------lk
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BAC_KFB       sawksla-ddkafraaqdgvndqvyyqpamersdnag---------lt
BAC_SUBT      sawksla-ndkefraaqdkvndhlyyqpamkrsdnag---------lk
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SAV6191       aawraea-gvaafraaqdaerdrvyfdpavrlarhdg---------lg
SCO0677       ddwrraadqdpqfrraqdderdrvyfdpavrrgkedg---------lr
STR_LIV       DDWRRAADQDPQFRRAQDDERDRVYFDPAVRRGKEDG---------LR
PSE_A01       qawadaa-etsefraaqdaerdrvyfdpavaqgkadg---------ls
STR_AM        nawrqaa-qdtvfqqtqeeerdrvyftpavsqakqdg---------lr
STR_AMBO      gdwqraa-gdpefrraqdderdrvyfgpavrqaqadg---------lr
STR_SCL       kdwrtaa-kdpafrkaqnderdrvyfdpavkqgkadg---------vg
STR_CLAV      RDWRKAA-GDEEFREAQNDERDRVYFNPAVQRGKSDG---------LG
CHR_VIOL      dkigslq-nnpawrdamwntfykvyiqysvqqarqrg---------fs
BAC_amMJ1_1   sawrslg-kdkdfraaqdtvndrlyyqpamkqsdnig---------lk
BAC_amFZ      sawrslg-ndkafraaqdkvndslyyqpamersenag---------lk
PAENIBAC      rkignlq-ddpewreamwktfyniyikyrgeqarnrg---------fn
PAE_EHIM      gkikklq-ndpawrkamwetfynvyirysveqarqrg---------ft
MICROB        qawadaa-etsefraaqdaerdrvyfdpavaqgkadg---------ls
REN_SALM      dawhqaa-kdsvflkeqdklrdsmyfnpavsqgksdg---------ls
BAC_DAU       sawrslg-ndkafraaqdkvndslyyqpamkrsenag---------lk
CBEI_0949     KDV-KAC-NDSLFKKAQLYELDEMYWKPAVSLATSIG---------AK
CHV_CVK2      kvinglg-ddkewqtavwdiyvklywtfaadfsdktgsaknrpgpvmt
CHV_PBU       kvinglg-ddkewqtavwdiyvklywtfaadfsdktgsaknrpgpvmt
PLAV_0939     nllkeag-adpvmrdtqdaffdrvywtpaaasairlg---------lt
CELL_JAP      nilrata-ddhimretqdlffdqaywqpaeraatqlg---------ik
DARO_2852     nllrata-ddpvmretqdlffdevywqpaakaadnfg---------ik
DARO_2340     nllrata-ddpvmretqdlffdevywqpaakaadnfg---------ik
NPUN_R2009    ekvhaia-ndqkwwkaqwdayistywqptlkllkskn---------ym

cons                                *
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NOC_N106        -algqfayydaivmhgpgdssdsfggirkaamkka-ktpaqgrdeaty
AMY_CS02        -algqfvyydaivmhgpgsssdsfggiraaamkka-ktpaqggdeaty
BAC_amMJ1       talakavmydtiiqhgggddpdslnslikrtnkkaggspkngvdekkw
BAC_CIRC        savtigsfvdtalnqgatggsdtlqgllars--------gsssnektf
BAC_KFB         talaravmyhtvrqrgdgddgdsryalikrtpkgaggspkegideqkc
BAC_SUBT        talaravmydtviqhgdgddpdsfyalikrtnkkaggspkdgidekkw
BUR_GLAD        saltigsfvdtalnqgadggsntlqgllsrs--------gnstdektf
SAV2015         -vlgqftyydaivmhgdggdstsfssirgralaka-eppaqggnevty
SAV6191         -algqfiyydamvvhgpgtdphslqgiraralrea-dtpaeggsetry
SCO0677         -tlgqfayydamvmhgdggg-lgfgsireralgra-rppaqggdevay
STR_LIV         -TLGQFAYYDAMVMHGDGGG-LGFGSIRERALGRA-RPPAQGGDEVAY
PSE_A01         -algqfvyydtlvvhgpgsqrdafggiraealsaa-lppsqggdetey
STR_AM          -algqfayydaavmhgesg----frdirsvalsra-lppsrggdekay
STR_AMBO        -tlgqfayydaivmhggggdrlgfgsirdralgra-rppagggdevay
STR_SCL         -vlgqfayydaivmhgggndatsfgsirkkalaka-eppaqggdekty
STR_CLAV        -VLGQFIYFDALVMHGDGDDSASFSSMRRKALAKA-KSPAQGGSEKTY
CHR_VIOL        saltigsfvdtalnqgatgdsgtlqgllsrs--------gnsgdektf
BAC_amMJ1_1     talakavmydtiiqhgggddpdslnslikrtnkkaggspkngvdekkw
BAC_amFZ        talakavmydtviqhgdgddpdsfyalikrtnkkmggspkdgtdekkw
PAENIBAC        saltigsfvdtalnhgatggseslqgllsks--------gsstdektf
PAE_EHIM        saltigsfvdtalnqgatgdsntlqgllars--------gsstnektf
MICROB          -algqfayydtlvvhgpgsqrdafggiraealsaa-lppsqggdetey
REN_SALM        -nlgqfmyydaifmhgpgdssdsfggirksamkna-ktpaqggdekty
BAC_DAU         talakavmydtviqhgdgddpdsfyalikrtnkkmggspkdgtdekkw
CBEI_0949       HQLTLAFIYDMCVNHGEDGAKEYINSAK----KKLGGLPKDGIDENKF
CHV_CVK2        spltrgfmvdvalnhgsn--mesfsdilkrmkn------kdekdeakw
CHV_PBU         spltrgfmvdvalnhgsn--mesfsdilkrmkn------reekdeakw
PLAV_0939       ealslaivydsvv-hgs------wislrdrtlaka-gqpsk-ageraw
CELL_JAP        tplgvavvydstv-hgs------aklirdktnqsa-gtlas-lgeqkw
DARO_2852       salgvavvydswv-hgs------wktlrdmtnqks-gpiat-lgeraw
DARO_2340       salgvavvydswv-hgs------wktlrdmtnqks-gpiat-lgeraw
NPUN_R2009      kaltigvlidtamnagmeddnsenwgveh-lftea---sddtdneedf

cons              :     .    *                        .*
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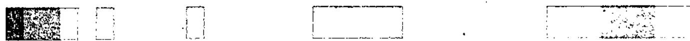
Q241

```
STR_N174      lnafldarkaamlteaahd-------dtsrvdteqrvflkagnldlnp
NOC_N106      lkaflaarktvmlkeeahs-------dtsrvdteqtvflnaknfdlnp
AMY_CS02      lnafldarkvimkqeeaha-------dtsrvdteqrvflnaknfdlnp
BAC_amMJ1     lnkfldvryddlmnpadpdtrdewresvarvdvl-rsiakannynlng
BAC_CIRC      mknfhakrtlvvdtnkynk---pp-ngknrvkqw-dtlvdmgkmnlkn
BAC_KFB       lnkfshvryddlmngamhdrrdewresvgrvhvl-rsianqnnynlng
BAC_SUBT      lnkfldvryddlmnpanhdtrdewresvarvdvl-rsiakennynlng
BUR_GLAD      mtsfyaqrtkvvdthdfnq---pp-ngknrvkqw-stlmsqgitslkn
SAV2015       lnafldarvwamrqeeahs-------dtsrvdtaqrvfltkgnlnldp
SAV6191       ldafldvrrtvmksekshh-------dttridtaqrrflydgnldlrt
SCO0677       lhafldervwamkqeqahs-------dtsrvdtaqrvflnegnldlep
STR_LIV       LHAFLDERVWAMKQEEAHS-------DTSRVDTAQRVFLNEGNLDLEP
PSE_A01       leaffdarnvimreepaha-------dtsridtaqrvflqngnfdler
STR_AM        lkafldareeemrkeeahs-------dttrvstaqrrflnegnldlnt
STR_AMBO      lhafldarvwamkqeeahs-------dtsrvdtaqrvflregnldldp
STR_SCL       lsafldarvwamkqeeahs-------dtsrvdtaqrvfltkgnlnldp
STR_CLAV      LNAFLDSRVWAMKQEAAHE-------DTSRVDTAQRVFVRQGNFALNT
CHR_VIOL      mtafyaqrskivdtndynq---pp-ngknrvkqw-stllnmgetdlkn
BAC_amMJ1_1   lnkfldvryddlmnpadpdtrdewresvarvdvl-rsiakannynlng
BAC_amFZ      lnkfldvryddlmnpsdedtqdewresvarvdvf-rdivkeknynlng
PAENIBAC      mtkfykertkivdtneyns---pp-ngknrvkqw-snllnmgetdlkn
PAE_EHIM      lkkfhakrtlvvdtneynq---pp-ngknrvkqw-dtlldmgkmnlkn
MICROB        leaffdarnvimreepaha-------dtsridtaqrvflqngnfdler
REN_SALM      lqafatarkkimkqenahs-------dtsrvddaqlkflnegnydlht
BAC_DAU       lnkfldvryddlmnpsdedtqdewresvarvdvf-rdivkeknynlng
CBEI_0949     LSSVMDYRYAFLKDDD-P-------EGSDRVNAY-RKLLKAGNVDLKP
CHV_CVK2      fldfcetrrkllksgfqdl---dtsktgdrailw-selfktgnvglkr
CHV_PBU       fldfcetrrkllkagfqdl---dtsktgdrctlw-anifkegnvglkr
PLAV_0939     slayvrerrnwlamhsn-t---llrktiyrmeafe-aliaaknwslal
CELL_JAP      ieayvatrrywlanhpr-k---dlrptvyrmdafq-rlidlnlwglel
DARO_2852     ikayvetrrswlanhsr-s---dlrgttyrmdafl-rlieqgywglal
DARO_2340     ikayvetrrswlanhsr-s---dlrgttyrmdafl-rlieqgywglal
NPUN_R2009    vdrfmelrlqfptr-dsg-------dmeerigaw-qkllrdrkwdmrv

cons               *                 *     :     :
```

```
STR_N174       pllwk--ty--------------------------------------
NOC_N106       plqwk--vy--------------------------------------
AMY_CS02       plqwk--vy--------------------------------------
BAC_amMJ1      plnvyseey--------------------------------------
BAC_CIRC       vdsei--aq--------------------------------------
BAC_KFB        plnvrshey--------------------------------------
BAC_SUBT       plnvrsney--------------------------------------
BUR_GLAD       c[illegible]--vk--------------------------------------
SAV2015        plewk--vy--------------------------------------
SAV6191        plewr--vy--------------------------------------
SCO0677        plewh--vy--------------------------------------
STR_LIV        PLEWH--VY--------------------------------------
PSE_A01        plews--vy--------------------------------------
STR_AM         plews--vy--------------------------------------
STR_AMBO       plewq--vy--------------------------------------
STR_SCL        plewk--vy--------------------------------------
STR_CLAV       PESWR--VY--------------------------------------
CHR_VIOL       adaav--qk--------------------------------------
BAC_amMJ1_1    plnvyseey--------------------------------------
BAC_amFZ       plnvrssey--------------------------------------
PAENIBAC       adsav--lq--------------------------------------
PAE_EHIM       vdsei--aq--------------------------------------
MICROB         plews--vy--------------------------------------
REN_SALM       plqwk--vy--------------------------------------
BAC_DAU        plnvrssey--------------------------------------
CBEI_0949      PENFT--VY--------------------------------------
CHV_CVK2       plkcyngyw--------------------------------------
CHV_PBU        plkcyngyw--------------------------------------
PLAV_0939      pmtvrg[illegible]depalg[illegible]nlrltspr[illegible]e
CELL_JAP       plvvrgl[illegible]snttlnat[illegible]ydgpqpgtrtlsvqapllrgldirlvq
DARO_2852      plvvhge[illegible]slatlrampppgcydgpqpgtrsialgtplargldvrllq
DARO_2340      plvvhge[illegible]slatlrampppgcydgpqpgtrsialgtplargldvrllq
NPUN_R2009     dlkny--v---------------------------------------

cons
```



```
STR_N174       ----------------------------------------gdpyvi
NOC_N106       ----------------------------------------gdsyai
AMY_CS02       ----------------------------------------gdpyqi
BAC_amMJ1      ----------------------------------------g-dfv-
BAC_CIRC       ----------------------------------------vtdwe-
BAC_KFB        ----------------------------------------g-ngv-
BAC_SUBT       ----------------------------------------g-nfv-
BUR_GLAD       ----------------------------------------vtswt-
SAV2015        ----------------------------------------gdsyh-
SAV6191        ----------------------------------------geryr-
SCO0677        ----------------------------------------gdayh-
STR_LIV        ----------------------------------------GDAYH-
PSE_A01        ----------------------------------------gdqfs-
STR_AM         ----------------------------------------geafsl
STR_AMBO       ----------------------------------------gdsyh-
STR_SCL        ----------------------------------------gdpyh-
STR_CLAV       ----------------------------------------GDSYH-
CHR_VIOL       ----------------------------------------vtnwe-
BAC_amMJ1_1    ----------------------------------------g-dfv-
BAC_amFZ       ----------------------------------------g-nft-
PAENIBAC       ----------------------------------------vtdwe-
PAE_EHIM       ----------------------------------------vtnwe-
MICROB         ----------------------------------------gdqfs-
REN_SALM       ----------------------------------------gdpye-
BAC_DAU        ----------------------------------------g-nft-
CBEI_0949      ----------------------------------------GDTFNI
CHV_CVK2       ----------------------------------------gkniv-
CHV_PBU        ----------------------------------------gkniv-
PLAV_0939      aalvkegvaiicdgvfdeqleiaiksfqqdygiiadgvaqpahrml-
CELL_JAP       lgisksgamlradgifgngswsawktfqtksmlpatgivsnaliakl-
DARO_2852      lgmsgrgvaikadgifgqtsvnllkrfqasqglpangiatpqgglsel-
DARO_2340      lgmsgrgvaikadgifgqtsvnllkrfqasqglpangiatpqgglsel-
NPUN_R2009     -----------------------------------------yi-
```

cons

```
STR_N174        ---ns
NOC_N106        ---ns
AMY_CS02        ---ng
BAC_amMJ1       ---ik
BAC_CIRC        ---mk
BAC_KFB         ---ik
BAC_SUBT        ---ik
BUR_GLAD        ---mk
SAV2015         ---ig
SAV6191         ---vp
SCO0677         ---ig
STR_LIV         ---IG
PSE_A01         ---ln
STR_AM          ---tg
STR_AMBO        ---vg
STR_SCL         ---ig
STR_CLAV        ---LD
CHR_VIOL        ---mk
BAC_amMJ1_1     ---ik
BAC_amFZ        ---iq
PAENIBAC        ---lq
PAE_EHIM        ---mk
MICROB          ---ln
REN_SALM        ---ik
BAC_DAU         ---iq
CBEI_0949       DGDVY
CHV_CVK2        ---is
CHV_PBU         ---is
PLAV_0939       ---gi
CELL_JAP        ---vs
DARO_2852       ---vd
DARO_2340       ---vd
NPUN_R2009      ---pq

cons
```

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