MODULATION OF VIRULENCE OF *STREPTOCOCCUS PNEUMONIAE* BY AN OPERON IN CONJUGATIVE TRANSPOSON Tn*5252*

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CHAPTER I

INTRODUCTION

Conjugative transposons are mobile genetic elements capable of intercellular transfer [1]. Apart from many similarities to conventional transposons, the mechanics of transfer of these elements resemble those of *E. coli* conjugative elements. As these elements are capable of conjugative transposition to various species of bacteria, they have been cited as the chief reason for emergence of multiple antibiotic resistance in Gram positive pathogens [2].

For several years, the research efforts in our lab have been focused on the biology of the streptococcal conjugative transposon, Tn*5252*, originally identified as a chromosome-borne element in *Streptococcus pneumoniae*. The left and right terminal regions have been characterized based on genetic, structural, and functional analyses of the DNA sequence. Several transfer-related genes were identified. These include a site-specific recombinase, excisionase, relaxase, a regulator of conjugative transfer, and several genes that are predicted to be involved in DNA transport. Interestingly, all these genes are clustered in the terminal regions of the element [3-8].

Compared to the left and right terminal regions, not much is known about the central 25 kb of Tn*5252* except that no apparent transfer-related genes are present in this region. Though the sequence of the central region of Tn*5252* has not been determined completely, we do have partial sequence data, at least one strand DNA sequence of this region. Recently, the genomic DNA sequence of clinical strain of *Streptococcus pneumoniae*, G54, was published [9]. A Tn*5252*-like element was identified in the genomic sequence. In the central region of this element, there exists a big operon, which shows significant similarity to our partial sequence data. All of these seem to imply that the big operon also exists in our transposon. In the operon in G54, there are 4 open reading frames. ORF2, the major part of the operon, encodes a large protein of 2074 amino acids, containing methylase and helicase domains. A blast search using the amino acid sequence of ORF2 also showed similar proteins with significant similarity not only in other species of *Streptococcus*, but also in *Enterococcus*, *Bacteroides*, *Vibrio*, *Clostridium* and so on. However the exact function of this protein is not known.

Specific aims proposed:

1. To carry out virulence assays to determine if the operon in the central region of Tn*5252* is related to the virulence of pneumococcus.
2. To determine whether this operon is inducible or constitutive by constructing a transcriptional fusion with the *E. coli lacZ* gene.
3. If this operon is related to the virulence of pneumococcus, determine the mechanism of its action.

CHAPTER II

REVIEW OF LITERATURE

Streptococcus pneumoniae (pneumococcus) belongs to the family *Streptococcaceae* and is a Gram positive α-hemolytic bacterium. Normally it is a commensal resident in the upper respiratory tract of 5-10 % of healthy adults and 20-40% of healthy children [10]. However, it can invade and cause minor to life-threatening diseases. In the United States of America, pneumococcus was annually responsible for about 50,000 cases of pneumonia, 3000 cases of meningitis and 7 million cases of otitis media annually before the introduction of the conjugate vaccine. Introduction of the conjugate vaccine has successfully decreased the rate of invasive disease from 21-33 cases to 13 cases per 100,000 in the population [11]. However, eradication of the diseases caused by pneumococcus is still far from expectations. Today, people at extreme ages and/or with a compromised immune systems are still at a much higher risk of invasive infection by pneumococci [12].

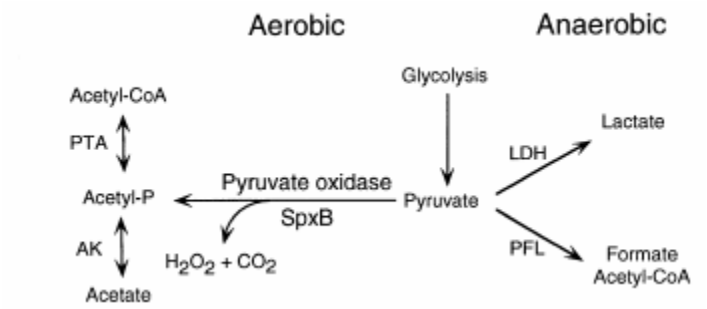
Pathogenesis of *S. pneumoniae*

Due to its significance in terms of its morbidity and mortality, pneumococcus has attracted significant research interest. In the past 100 years, there has been a lot of

improvement in the identification and characterization of pneumococcal virulence factors and virulence genes. However, the detailed mechanism of pathogenesis is still not fully elucidated. In the following narrative, most of the factors that are currently known to contribute to the survival of pneumococcus during invasion and infection will be reviewed.

Pyruvate oxidase

S. pneumoniae is a fermentative aerotolerant anaerobe. It has the common glycolysis pathway to break down carbohydrates ultimately to pyruvate. However, the tricarboxylic acid (TCA) cycle and electron transport chain are missing [10]. As shown in Fig 1, in anaerobic growth conditions, pyruvate is further degraded to lactate or formate [13]. Though pneumococcus prefers an anaerobic environment, it is able to survive and grow in an aerobic environment. In the presence of oxygen, pyruvate oxidase can catalyze the reaction to convert pyruvate to generate acetyl-P using oxygen [14]. Acetyl-P is involved in a further reaction to generate ATP[15]. This reaction is not only a method to degrade pyruvate and promote glycolysis, but more importantly, it is a reaction to detoxify oxygen and generate hydrogen peroxide, which is taken advantage of by pneumococcus to compete and inhibit other potential pathogens residing in the nasopharyngeal region [16]. A number of studies have shown that pyruvate oxidase is required for both nasopharyngeal and intraperitoneal infection. In particular, clinical strains carrying a mutation in pyruvate oxidase were found to be deficient in their capacity to attach to



Metabolic pathways. PTA, phosphotransacetylase; AK, acetate kinase; LDH, lactate dehydrogenase; PFL, pyruvate formate lyase.

Fig 1. Central metabolism pathway in *S. pneumoniae*. In aerobic environment, pyruvate is degraded by a reaction catalyzed by pyruvate oxidase. In an anaerobic environment, pyruvate degraded by a reaction catalyzed by lactate dehydrogenase or pyruvate formate lyase [14].

vascular endothelial cells and type II lung cells [14]. However, a detailed understanding of the role of pyruvate oxidase in pneumococcal pathogenesis is not yet available.

Capsule

The capsule of pneumococcus has been identified as a virulence factor for a long time. It is a thick polysaccharide layer residing outside of the pneumococcus cell-wall [17]. The lethal dose for encapsulated strains is only 50% of the non-encapsulated strains. Consistently, encapsulated strains are found to be about 10^5 more virulent than non-encapsulated strains [18]. So far, more than 90 different serotypes of capsule have been identified and there is no cross immune-reactivity between them [19]. Through blocking the access of cell wall localization by the complement system, the capsule ultimately blocks phagocytosis [20-22]. Different degrees of virulence have been observed with the various types of capsules. However, it has been shown that the capsule is not the sole determinant of pneumococcal virulence. The genetic background is also very important for the full expression of virulence, indicating that other possible factors are involved in pathogenesis [23].

Pneumolysin

A potent cytolysin, termed pneumolysin, has been observed to be present in every clinical isolate of pneumococcus. It is a cytoplasmic protein which is not automatically secreted outside after synthesized. The cytotoxic function of pneumolysin on host cells

thus is directly dependent on an another pneumococcal virulence factor, autolysin [22, 24]. Once pneumolysin has accumulated in high concentration, it oligomerises to form pores on mammalian cell membranes and causes cell lysis. Due to the action of the pneumolysin, ciliated bronchial epithelial cells are damaged and lose their normal function to clear mucus from the lower respiratory tract, which ultimately facilitates the spread of pneumococcal infections. Pneumolysin is also able to cause damage to alveolar epithelial cells, which enriches infected cells with nutrients from alveolar flooding. More importantly, disruption of the alveolar-capillary boundary facilitates penetration of pneumococcus through the epithelium and ultimately enterance into the blood stream [25-27]. At low concentrations, pneumolysin can still inhibit neutrophil and monocyte respiratory bursts, chemotaxis, production of lymphokines, and immunoglobulins [28].

Autolysin

Autolysin belongs to a large group of enzymes which have the ability to degrade the peptidoglycan backbone of bacterium. The regulated action of autolysin does not necessarily lead to complete lysis of a bacterial cell. Mostly autolysins are involved in bacterial cell wall synthesis, its turnover, and separation of cells during division [29]. Under laboratory conditions, autolysin, (encoded by *lytA*), is responsible for autolysis during stationary phase and competence development. As mentioned above, autolysis of pneumococcal cells during infection helps to release pneumolysin. Also, it has been reported that a host defense factor, lysozyme, can trigger the release of autolysin, and ultimately, help to release Pneumolysin [22, 30].

Pneumococcal surface protein A (PspA)

*Psp*A is located on the cell wall of pneumococcus. It displays structural and antigenic variability among different pneumococcal strains. During infection, *PspA* helps to inhibit complement activation to facilitate pneumococcal infection [22].

Pneumococcal surface antigen A (PsaA)

PsaA is a membrane protein, composed of 309 amino acids with a molecular weight of 34, 539 Da[22]. A *psaA*$^-$ mutant has been shown to be avirulent in both intraperitoneal and intranasal challenges model [31]. PsaA protein belongs to the ABC-transporter family with an ATP binding subunit. Sequence analysis suggests that it is responsible for the transfer of Zn^{2+} and Mn^{2+} from outside into the cytoplasm of a pneumococcal cell. Failure to acquire Zn^{2+} and Mn^{2+} during infection might be the real reason that *psaA*$^-$ mutants become avirulent in intraperitonial and intranasal challenges. Consistent with this observation, it has been shown that Mn^{2+} is not only required by superoxide dismutases (SOD) of pneumococcus to detoxify hydrogen peroxide generated during aerobic growth, it also serves as a signal which is required for the induction of several virulence related genes in pneumococcus within different host sites [32, 33]. The function of Zn^{2+} during pneumococcal infection is not very clear. It has been shown that zinc deficiency in the diet of mice will lead to low concentrations of Zn^{2+} in both femur and plasma. Ultimately the mice showed reduced immune response to *PsaA* antigen,

suggesting more extensive pneumococcal colonization and severe infection with an increased risk of death [34].

Hyaluronate lyase

Hyaluronate lyase, a surface protein of pneumococcus, belongs to a broader group of enzymes, named hyaluronidases. It is required for full virulence of pneumococcus. During infection, it is responsible for breaking down extracellular matrix components to increase tissue permeability. Mostly it is associated with pneumonia, bacteremia, and meningitis [22, 24, 35].

BgaA

In most other bacteria, β-galactosidase (BgaA) is the key enzyme responsible for lactose metabolism. However, in pneumococcus it is not involved in lactose utilization, since bgaA$^-$ mutants can still use lactose as the only carbon source [36]. Recently it was reported that BgaA, together with NanA and StrH, two exoglycosidases, contributes to attachment on the cell surfaces and initiation of colonization through cleavage of mammalian glycol-protein [37]. Expression of *BgaA* is induced by lactose, the biological significance of which is not clear yet.

Zinc metalloproteinase (Zmp)

According to the published genomic sequences of three pneumococcal strains, (R6, TIGR, and G54), at least two different kinds of zinc metalloproteinases are present in all strains. Strain R6 process *ZmpA*, an IgA-specific proteinase. It cleaves the hinge region of IgA, helping pneumococcus survive in the nasopharyngeal region. *ZmpB,* a virulence factor involved in both intraperitoneal and intranasal infection, can promote TNFα production in the host [38]. Besides these two zinc metalloproteinases in the R6 strain, the TIGR strain also carries *ZmpC*, a matrix metalloproteinase, which is involved in matrix remodeling during infection. Its effect in virulence is not as important as *ZmpA* and *ZmpB*. Besides the above three, in G54, there exists another zinc metalloproteinase, *ZmpD*, which is also involved in virulence. Its function and biological significance still remain to be determined [39, 40].

Two component signal transduction system

Two component signal transduction-systems (TCS) are employed by bacteria to sense various signals in the outside environment to adjust and regulate cellular processes accordingly. They are mostly composed of a membrane-associated sensor histidine kinase (HK) and a cytoplasmic congnate response regulator (RR). An external stimulus will activate HK and make it autophosphorylate itself. Then the phosphorylated HK will further phosphorylate RR. RR, mostly a DNA-binding transcriptional regulator, assumes a conformational change following phosphorylation, and as a result, a given set of genes

may be up-regulated or down-regulated. So far, 14 TCSs have been identified in pneumococcus, eleven of which have been shown to have roles in virulence [41]. Some of them, such as TCS04, have been shown to directly regulate already known virulence factors, while most of them are involved in complex regulatory networks [42]. The details of these global regulatory networks are yet to be determined.

Phase variation in pneumococcus

As has been mentioned, pneumococcus is able to colonize different niches with different ecological characteristics within the human host. The nasopharyngeal region is an aerobic environment, while most of the other places provide anaerobic environments. Pneumococcus has developed a mechanism involving phase variation to adapt to these different environments. Phase variation in pneumococcus has been correlated with variations in colony morphology: transparent and opaque [43]. Each phase has a different profile of gene expression inside the cell. Under laboratory conditions, a clinical isolate can spontaneously change its colony morphology at a frequency ranging from 10^{-5} to10^{-7}. Infection experiments have shown that transparent variants are able to attach to human vascular endothelial cells and cytokine-stimulated type II lung cells with high efficiency [43]. As a result, they are able to establish into more dense and stable colonies on mucosal surfaces of the nasopharynx. They also efficiently bind to platelet-activating factor receptor which allows the bacterial cell cross these cells and the blood-brain barrier more efficiently [44]. On the other hand, opaque variants are able to more efficiently cause systematic infections after intraperitoneal inoculation into mice[45]. It is suggested

that such increased virulence is due to decreased opsonophagocytic killing of opaque pneumococci [46]. This has been shown by in vitro experiments. By comparing the expression profiles of transparent and opaque variants, it has been determined that expression of pyruvate oxidase and a putative proteinase maturation protein homolog are increased while expression of a putative elongation factor, Ts, is decreased [47]. However, the detailed mechanism of how phase variation happens during infection and how it is regulated are not fully understood.

Treatment of infections due to pneumococcus

The conjugate vaccine based on pneumococcal capsular polysaccharide serotypes, is still not as successful as expected. Many groups of individuals have a poor immune response to one or more than one kind of capsular polysaccharide in the vaccine, especially people of extreme ages [48, 49]. Conjugative vaccines, in which smaller numbers of polysaccharides are chemically conjugated to non-pneumococcal proteins, have improved the immunogenity of the polysaccharides. However, the conjugative vaccine still has only a limited effect, since pneumococcus periodically goes through modifications of the capsule. Moreover, the high costs involved in making the vaccine are still a factor limiting its widespread use [24, 50].

Antibiotics are still the best way to treat the diseases caused by pneumococcus. With indiscriminate use of antibiotics in some communities, pneumococcus has gradually gained resistance. In the 1960s, almost all clinical isolates of pneumococcus were

sensitive to β-lactam antibiotics. Currently, β-lactam resistance in pneumococcus is quite common. In 2000, more than 15 % of clinical isolate show complete resistance to penicillin. Some strains of β-lactam resistance pneumococcus also show resistance to multiple antibiotics such as erythromycin, macrolides, and clindamycin. These factors underscore the need and urgency for an enlarged understanding of the biology of this human pathogen [51-54].

Conjugative Transposon

Conjugative transposons are responsible for the dissemination of multiple antibiotic resistance among pneumococci

Emergence of multiple antibiotic resistances in pneumococcus was initially thought to be associated with plasmids. However the efforts to identify the related plasmids were not successful [55-58]. Though pneumococcus is able to accept and maintain plasmids from other streptococci, no plasmids have been discovered in most of the clinical isolates. On the other hand, Shoemaker et al. [59] showed that antibiotic resistance determinants were part of a chromosome-borne genetic element. This element was able to transfer within the same and between different species of streptococci by a process requiring direct cell-to-cell surface contact. Since the whole process was resistant to DNase, the possibility of transformation was eliminated. This large fragment of mobile DNA was named a conjugative transposon.

Conjugative transposons which are quite different from most common transposons have a completely different mechanism of transposition. It is named "conjugative" because the transferred molecule originates in a different cell from the recipient. Cell-to-cell direct surface contact is required for the initiation of transposition [2]. The transposon then excises itself out of the chromosome to form a circular intermediate. With the help of a DNA relaxase, one strand is relaxed from the circular intermediate and further transferred into recipient cells through a mating structure formed by the conjugative transposon. In the recipient cells, the newly received DNA recircularize before it integrates into the chromosome in a reaction requiring the activity of the integrase encoded by transposon [1].

Classification of Conjugative Transposons

DTnERL/CTnDOT family of conjugative transposons in *Bacteroides*

Conjugative transposons identified in *Bacteroides* vary in size from 45 kb to 150 kb[60]. There are 5 to 8 preferred sites in the *Bacteroides* chromosome at which conjugative transposons insert. Most transposons identified in *Bacteroides* fall into two groups: DTnERL and CTnDOT, both of which have the same regulatory backbone except that CTnDOT contains an extra 13 kb DNA which renders resistance to the macrolide-lincosamide-streptogramin group of antibiotics [61]. All transposons belonging to this group share a conserved sequence at the and right ends while the sequence in the center varies. The presence of subinhibitory concentrations of

tetracycline can induce excision of the transposon 100~1000 times more frequently and, in this case, self-transfer of the transposon to other loci in the chromosome results in the absence of any mating partners [62].

When the conjugative transposon transposes, three other kinds of elements can be co-transferred at the same time. First, a conjugative plasmid in the donor cells is able take advantage of the mating pore formed by conjugative transposon during transfer. In this case, conjugative plasmid will use its own machinery to nick at its own *oriT* and initiate conjugative transfer[62]. Second, the non-replicating *Bacteroides* unit (NUB) can also be co-transferred during conjugative transfer. In comparison to DTnERL/CTnDOT, NUBs are smaller in size. Sequence alignment shows that NUB has a significant similarity to DTnERL/CTnDOT except that they do not possess the regulatory genes required for transfer related genes. When DTnERL/CTnDOT gets activated for transfer, NUB can take advantage of the integrase and excisionase encoded by DTnERL/CTnDOT to excise out of the chromosome and form a circular intermediate. The NUB then uses its own protein, *mob*, to nick at *oriT* that relaxes one strand out. The same as DTnERL/CTnDOT, NUB need the mating pore formed by conjugative transposon to transfer the relaxed strand into the recipient cells [63]. Third, the mobilizable transposon Tn*4399* can also be co-transferred along with DTnERL/CTnDOT. Tn*4399* transfers by the same mechanism as NUB, in a way that Tn*4399* requires the integrase and excisionase system and mating pore system of the conjugative transposon for its own transfer. However, the detailed mechanism is not clear yet. There are two genes, *mocA* and *mocB*, in Tn*4399* that are

required for mobilization of Tn*4399*, but no circular intermediate has been identified so far [64].

Conjugative Transposons in *Enterobateriaceae*

So far, there are three groups of conjugative transposons identified in *Enterobacteraceae*. They are R391, belonging to incompatibility J group (IncJ), isolated from *Proteus rettgeri* [65]; CTnscr94 isolated from *Salmonella senftenberg* [66]; and the SXT element isolated from *Vibrio cholera* 0139 [67]. All of these three elements contain the λ-type integrase system. Though there is evidence of independent conjugative transfer in each element, the detailed mechanisms are yet to be determined. Study of pathogenicity islands shows that enteric pathogenicity islands have similar regulatory backbone structures similar to these conjugative transposons[68].

Tn*916*: A Model to Study Mechanism of Transposition

Tn*916* was originally discovered in the hemolytic multidrug-resistant *E. faecalis* DS16 [69]. It is an 18 kb element carrying *tetM*, which renders the host tetracycline resistance. An interesting feature of this element is that the presence of subinhibitory concentrations of tetracycline can increase the conjugation efficiency more than 1000-fold [70]. It was discovered that a number of conjugative transposons from various sources also possess *tetM* and similar regulatory genes, which implies a similar mechanism of transposition shared by these elements. For this reason, Tn*916* has been

used as a model to study the regulation of transposition. All the genes required for integration, exicision and regulation of integration and excision are located near the junctions. Integrase is responsible for the excision of the transposon from the chromosome in the donor and site-specifically inserts the element transposon into the chromosome of the recipient [70]. Complementation experiments showed that an active *int* gene in the donor cell was sufficient for the whole process of conjugal transfer, implying that the integrase function in the recipient was actually provided by the donor cells[71]. The gene encoding Xis is located directly upstream of *int* and is required for excision to form circularization of the element in the donor. It can bind to both junctions of the transposon, with higher affinity for the left junction and lower affinity for the right junction. When there is a just sufficient amount of excisionase, it prefers to bind to the left junction which stimulates the excision function. When there is an excess, excisionase begins to bind to the right junction which represses excision [72, 73]. Different from that of *E.coli* λ phage in which an integration host factor (IHF) plays an important role in facilitating integration and excision, the IHF has very little effect on excision and integration of Tn*916*. It has been reported from different sources that excision of Tn*916* happens more than 1000-fold in *E.coli* in comparison to a Gram-positive host. Originally this was explained by expression of *int* and *xis* in Tn*916* being loosely regulated in *E.coli*. However, over expression of *int* and *xis* did not increase excision of Tn*916* to the expected level in a gram positive organism. Recently it was explained that, in *E. coli*, histone-like factor (HU) greatly enhances excision probably by enhancing the integrase-excisionase-DNA structure formed during excision [73].

Tn*5252*

Conjugative transposon Tn*5253* was originally discovered in a clinical isolate of *S. pneumoniae*, BM6001. It was shown to be a composite structure of two independent conjugative transposons, Tn*5251* which showed extensive similarity to Tn*916*, and Tn*5252*, a unique type of element. Tn*5252* recognizes a specific *att* site of 35 bases for genetic integration. After insertion, the *att* site is duplicated and flanks the transposon as direct repeats [4].

Transfer-related Genes in Tn*5252*

By DNA sequencing, genetics and biochemical analyses, most of the transfer related genes of Tn*5252* were shown to be located in the two termini of the transposon. Similar to the structure of transposon Tn*916*, at the left end there are *int* and *xis* which are responsible for integration and excision of transposon during conjugal transfer [4]. In the left end, there is another operon with three open reading frames, orf4, orf9 and orf10. Though the exact functions of orf9 and orf10 are not known, orf4 has been found to encode a protein with a DNA relaxation function. It is thought to bind to the *oriT* in the circular form of transposon and to make a nick during transposition[6]. Also on the left side, there is orf3 which encodes a protein with putative regulatory function. An insertion mutation in orf3 completely abolishes conjugal transfer during mating. On the other hand, in the mutant strain the function of orf3 cannot be complemented in trans by providing a plasmid carrying the intact orf3 [7]. On the right junction, an operon with several open

reading frames is present. The deduced amino acid sequence of these open reading frames show high similarity to membrane mating components formed by conjugative plasmids during conjugation, which implies that these open reading frames probably encode the protein products for channel formation required for conjugative transposition of the element [8].

Non-transfer Related Genes in Tn5252

At the right junction, there is a gene encoding cytosine methylase that is constitutively expressed. Insertion mutations within this gene do not have detectable influence on conjugal transposition [74]. The biological significance of this gene is still not known. In the central region of the transposon next to relaxase operon, there is an operon with four open reading frames. The deduced amino acid sequence of one of these shows high similarity to the *umu* protein which is responsible for DNA damage repair in the SOS system. It has also been shown that this operon gives the bacterium resistance to higher doses of UV light [75].

Also in the central region of the transposon, there is a *cat* gene which is able to give pneumococcus resistance to chloramphenicol. It has been shown that the *cat* gene in Tn*5252* is flanked by an *IS*-like sequence. Spontaneous recombination between the *IS*-like sequences flanking the *cat* gene would make the host cell lose resistance to chloramphenicol by the "curing" of this segment of DNA. This instability of *cat* in Tn*5252* implies that the significance of Tn*5252* to pneumococcus might not be to provide

chloramphenicol resistance, as traditionally suggested. On the other hand, determinants of the actual advantage rendered by Tn*5252* rely on further analysis on the non-transfer related genes in central region. In the work presented following, we studied the function of an operon with 4 open reading frames, the majority part of which encode a DEAD-box protein with 2074 amino acids having a molecular weight of 239.8 KDa. Virulence assays indicate that this operon is able to modulate the virulence of pneumococcus depending on the route of infection. Following that, we carried out efforts to determine the effect of this operon on other virulence related genes or virulence related process.

CHAPTER III

METHODOLOGY

Bacterial Strains

All the non-encapsulated avirulent *S. pneumoniae* strains used in this study were derived from the non-encapsulated wild type strain, Rx1 [76]. The detailed genotype of each strain is listed in Table I. All the *E. coli* strains used in this study are listed in Table I.

Growth and Storage of Bacterial Strains

Medium for *S. pneumoniae*

Casein hydrolysate (CAT) medium was used to grow all pneumococcal strains unless mentioned otherwise. The CAT medium was made by dissolving 10 g of casein hydrolysate, 5 g tryptone, 5 g NaCl and 1 g yeast extract in one liter of distilled water. After sterilization, glucose and K_2HPO_4 were added to yield a final concentration of 11 mM and 16 mM, respectively. The full medium is named CATPG. If needed, 1.5 % (w/v) of agar was added to the medium. Unless mentioned otherwise, pneumococcal cultures

were grown statically in slip cap tubes at 37 ℃. To aerate pneumococcal cultures, a 1~2 ml of a pre-grown static culture was placed into a 75 ml of conical flask, incubated at 37 ℃, and shaken at a speed of 200 rpm. For storage, all the pneumococcal cultures were grown to $OD_{550\ nm}$=0.2-0.3, supplemented with 10% (v/v) sterile glycerol and stored at -80 ℃.

Chemically Defined Medium

If needed, a chemically defined medium modified from Van de Rijn was used to culture pneumococcal cells [87]. The detailed recipe is shown in Table II.

Medium for *E. coli*

Luria-Bertani (LB) medium was used to grow all *E.coli* strains. The medium was made by dissolving 10 g of tryptone, 5 g yeast extract, and 10 g NaCl into one litter of distilled water. The pH of the medium was adjusted to 7.5 by NaOH before sterilization. Unless mentioned otherwise, *E.coli* cultures were grown in a shaking incubator at 37 ℃ at 200 rpm. If needed, 2 % (w/v) of agar was supplemented into medium. For short-term storage, overnight cultures were stored at -20 ℃ with 50% sterile glycerol while for long-term storage, they were kept at -80 ℃ with 10 % sterile glycerol. Antibiotics were supplemented into the medium if needed. The concentrations of antibiotics used for selection during culturing bacteria are listed in Table III.

TABLE I. Bacterial Strains and Plasmids

Strains or Plasmids	Description	Source or reference
S. pneumoniae strains		
D39	Type 2 encapsulated, virulent	[77]
Rx1	Non-encapsulated laboratory strain	
CP1250	*malM511 str-1 β-gal*$^-$	[78]
SP1000	*str-1* Tn*5252* Em Cm	[79]
SP1400	SP1000 derivative *β-gal*$^-$ Tn*5252*	This study
SP1403	SP1400 derivative, Tn*5252*Δ *cat*	This study
SP1407	SP1403 (pVJ572 Tc^r)	This study
SP1460	SP1407 derivative, Pm *malX* – Em^r cassette inserted upstream of the helix operon	This study
SP1471	SP1403 derivative, Pm *malX* – Em^r cassette inserted upstream of the helix operon	This study
SP1472	SP1403 (ORF2 of helicase operon Ω *lacZ*)	This study
SP1476	SP1471 (ORF2 of helicase operon Ω *lacZ*)	This study
SP1519	SP1403 derivative, Pm *malX* – Em^r cassette inserted upstream of the helix operon	This study
SP1525	SP1403 derivative, *spxB* Ω *lacZ*	This study
SP1528	SP1519 derivative, *spxB* Ω *lacZ*	This study
SP3001	*str-1,* derivative of D39	This study
SP3003	*nov^r* derivative of D39	This study
SP3004	SP3001ΩpSS141 carrying the helicase operon Em^r	This study
SP3005	SP3004 (ORF2 of helicase operon Ω Km^r)	This study
SP3100	*nov^r* derivative of WU2, an encapsulated serotype III clinical strain	This study
SP3101	*str-1* derivative of WU2	This study
SP3102	SP3101ΩpSS141 Em^r	This study
SP3103	SP3102 (ORF2 of helicase operon Ω Km^r)	This study
WU2	Type encapsulated, virulent	[80]
E.coli strains		
XL1 Blue MRA Stratagene		
Plasmids		
pDG647	Amp^r Em^r	[81]
pDL278-D	Spc^r	[82]
pDR18	pSK+::1.65 kb *Hin*dIII fragment from within ORF2 of the helicase operon	This study

pEVP3	Cm^r	[83]
pLS1	Tc	[84]
pRK1301	pACYC184::4 kb DNA carrying promoterless *lacZ* of *E. coli*	Jacques Fresco
pSJ170	Amp^r Km^r	[85]
pSS141	pVA891::28.1kb *Kpn*I fragment from Tn*5252*	[5]
pVA891	Cm^r Em^r	[86]
pVJ572	pLS1 (*Eco*RI)::1.1 kb PCR amplified DNA carrying *malR* of Rx1	This study
pVJ588	pDL276-D (*Sma*I)::0.6 kb PCR-amplified DNA using primers Pm-mal3/Pm-mal4 and carries the pneumococcal *malX/M* promoters	This study
pVJ595	pVJ588 (*Hin*dIII)::1.6kb *Hin*dIII Em^r fragment of pDG647	This study
pVJ604		
pVJ605		
pZH2	pSJ170 (*Bgl*II):: 4 kb *Bgl*II DNA fragment carrying *lacZ* from pRK1301	This study
pZH12	pDR18 (*Bgl*II):: 5 kb *Bam*HI DNA fragment containing the *lacZ*-Kan^r cassette from pZH2	This study
pZH38	pEVP3 (*Sma*I)::1 kb DNA amplified using the primers, G54-1 and G54ORF1-F	This study
pZH54		

Table II. Recipe of modified chemically defined medium

Components	Concentration (mg/L)
$FeSO_4 \cdot 7H_2O$	5
$Fe(NO_3)_2 \cdot 9H_2O$	1
K_2HPO_4	200
KH_2PO_4	1000
$MgSO_4 \cdot 7H_2O$	700
$MnSO_4$	5
L-Alanine	100
L-Arginine	100
L-Aspartic acid	100
L-Cystine	50
L-Glutamic acid	100
L-Glutamine	200
Glysine	100
L-Histidine	100
L-Isoleucine	100
L-Leucine	100
L-lysine	100
L-Methionine	100
L-Phenyalanine	100
L-Proline	100
L-Hydroxy-l-proline	100
L-Serine	100
L-Threonine	200
L-Tyrptophan	100
L-Tyrosine	100
L-Valine	100
p-Aminobenzoic acid	0.2
Biotin	0.2
Folic acid	0.8
Niacinamide	1
β-Nicotinamide adenine dinucleonide	2.5
Pantothenate calcium salt	2
Pyridoxal	1
Pryidoxamine dihydrochloride	1
Riboflavin	2
Thiamine hydrochloride	1

Vitamine B12	0.1
Glucose	10000
Adenine	20
Guanine hydrochloride	20
Uracil	20
$CaCl_2 \cdot 6H_2O$	10
$NaC_2H_3O_2 \cdot 3H_2O$	4500
L-Cysteine	500
$NaHCO_3$	2500
$NaH_2PO_4 \cdot H_2O$	3195
Na_2HPO_4	7350
Yeast extract	0.04% (w/v)

Chemicals

Chemicals were purchased from Sigma (USA) unless mentioned otherwise.

Chromosomal and Plasmid DNA Isolation

Chromosomal DNA isolation from pneumococcus

To isolate chromosomal DNA, pneumococcal cultures were started by inoculation of 1 ml culture stored at -80 ℃ into 100 ml CATPG, with proper antibiotics if needed until $OD_{550\ nm} \approx 0.3$. The cells were collected by centrifugation at 5,800 g in a Sorvall GSA rotor at 4 ℃ for 10 minutes. After discarding the supernatant, the pellets were resuspended in 4 ml of 10 mM ethylenediamine tetraacetic acid (EDTA) (pH=8.0) and kept on ice for 10 minutes. To initiate lysis, 3 ml of lysis solution [30mM EDTA, 0.1 % (w/v) sodium dodecyl sulfate (SDS), 0.4 % sodium deoxycholate (DOC), and 100 µl RNase], was added. The lysis mixture was kept at 37 ℃ until the solution became clear. Then, proteinase K was added to a final concentration of 100 µg/ml to degrade nucleases. The solution was incubated at 65 ℃ overnight. The DNA was extracted at room temperature with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) for 10 minutes, twice with chloroform-isoamyl alcohol (24:1) for 10 minutes and finally precipitated with two volumes of 95 % ethanol at -20 ℃ for at least 20 minutes. The DNA pellet was collected by centrifugation at 6,500 g in a Sorvall GSA rotor for 20

minutes. Then the DNA pellet was vacuum dried (Forma-Vac, Forma Scientific, Inc.), before it was resuspended in 1 ml TE buffer [10 mM Tris-Cl (pH 7.5), 1 mM EDTA]. The DNA concentration was determined using a UV spectrophotometer (Spectronic 1001, Milton Roy Co.).

Plasmid DNA isolation from *E.coli*

Plasmid DNA isolation was done by using a Wizard column (Promega). Briefly, 3 ml of overnight culture was pelleted by centrifugation at 15,000 g for 30 seconds in a bench top centrifuge (Beckman). Cells were resuspended in 0.3 ml resuspension solution [25 mM Tris-HCl (pH=8.0), 10 mM EDTA, and 25 μg/ml RNase]. Cells were lysed by addition of 0.3 ml of lysis solution (0.2 M NaOH, 1 % SDS) and kept at room temperature for 5 minutes. Then the lysate was mixed with 0.3 ml of neutralization solution [3 M potassium acetate (pH=5.2)] and kept on ice for 10 minutes. The lysate was centrifuged at 15,000 g in bench top centrifuge machine for 10 minutes. The supernatant was saved and mixed with resin [4 M guanidine thiocyanate, 50 mM Tris-HCl (pH=7.0), 10 mM EDTA, 10 mM diatomaceous earth] before being passed through a Wizard column. The column was washed with 3 ml of column washing solution [100 mM NaCl, 10 mM Tris-HCl (pH=7.5), 2.5mM EDTA and 50% ethanol (v/v)]. For DNA sequencing templates, a special washing solution [50% ethanol (v/v), 10 mM Tris-HCl (pH=7.4), 10 mM KAc, 1μM EDTA] was applied. Then samples are centrifuged at 15,000 g for 30 seconds to completely remove the washing solution remaining in the column, 50-100 μl

Table III

Selective Antibiotic Concentrations

		Concentrations (µg/ml)	
Genotype/Phenotype	Antibiotic	Stab Plate/Broth	Overlay
Streptococcus pneumoniae			
cat (Tn5252)	chloramphenicol	5	15
Em^r	erythromycin	3	5
nov	novobioicin	10	10
str	streptocmycin	200	200
aphA (Tn*1545*)	kanamycin	200	1000
tet (pLS1)	tetracycline	1	1
Cm^r (pEVP3)	chloramphenicol	3	5
$spec^r$	spectinomycin	250	350
E.coli			
Em^r	erythromycin	500	
Kam	kanamycin	50	
Cm^r	chloramphenicol	15	
Amp^r	ampicillin	50	
$Spec^r$	spectinomycin	150	

of preheated TE was added into column, and incubated 1 minute before a final spin at 15,000g for 1 minute to elute the DNA from the column.

Plasmid DNA isolation from pneumococcus

To isolate plasmid DNA, 20 ml of pneumococcal cells, pre-grown under selective pressure to $OD_{550\ nm} \approx 0.3$, was pelleted at 6,500 g in Sorvall GSA rotor at 4 ℃ for 10 minutes. The cells were resuspended in 0.3 ml of resuspension solution. Then the following steps were the same as isolating plasmid from *E.coli* using the Wizard columns.

Transformation

Pneumococcal Transformation

For transformation, recipient cells (10^6 CFU), donor DNA (1μg/ml for chromosomal DNA and 10 μg /ml for plasmid DNA), and 100 ng cps-1 (competence inducing factor) were mixed into 1ml of CTM [CATPG with 0.2% (w/v) bovine serum albumin (BSA) (USB), and 1 mM $CaCl_2$]. The mixture was kept at 37 ℃ for 90 minutes before addition of DNase I at a final concentration of 25 μg/ml to stop transformation. Various dilutions of the transformation mixture were added into 4 ml of CATPG broth with 0.1% (w/v) BSA (Fisher). Then 4 ml of agar, pre-cooled to 49 ℃, was mixed with the broth containing the transformation mixture and poured on top of a 20 ml base

CATPG agar. After the cell layer solidified, 10 ml of CATPG agar was overlaid (buffer layer) and incubated for 90 min at 37 ℃ for phenotypic expression. The plate was then overlaid with 10 ml CATPG agar containing the appropriate antibiotic and incubated at 37 ℃ overnight. If needed, 50µl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), dissolved in dimethylformide (40mg/ml), was spread on the surface of base agar prior to overlaying cell layer to identify β-galactosidase$^+$ cells.

E.coli **Transformation**

E.coli competent cells were prepared and transformed according to method of Hanahan [88]. To prepare frozen competent cells, 0.5 ml of overnight *E.coli* culture pre-grown in SOB [2 % (w/v) bactotryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, and 10 mM $MgSO_4$] was inoculated into 50 ml of SOB. After precisely two and half hours, the culture was transferred to ice and incubated for 15 minutes. Cells were then pelleted at 5,800 g for 10 min at 4 ℃. The supernatant was discarded and cells were resuspended in 8 ml of ice cold FSB [10 mM KAc, 100 mM KCl, 45 mM $MnCl_2$, 10 mM $CaCl_2$, 3 mM hexamine cobalt and 10 % (v/v) redistilled glycerol]. The cells were kept on ice for 15 minutes and then pelleted again at 5000 g for 10 minutes at 4 ℃. The supernatant was discarded and cells were resuspended in 2ml of FSB. After addition of 70 µl of DMSO, the cells were kept on ice for 5 minutes, another 70 µl of DMSO added, and incubated on ice for another 10 minutes. The cells were dispensed in volume of 200 µl volume into pre-chilled eppendoff tubes and stored at -80 ℃.

For transformation, the frozen competent cells were thawed on ice. After addition of donor DNA (less than 10 µl in volume per 200µl of cells), the mixture was kept on ice for 30 minutes. The cells were given a heat shock at 42 ℃ for 90 seconds and transferred to ice immediately for 2 minutes. After addition of 800 µl of SOC (20 mM glucose in SOB), the cells were transferred to shaking incubator at 37 ℃ for 1 hour phenotypic expression. Finally cells were spread on LB plate with the appropriate selective antibiotic. Plates were incubated at 37 ℃ for 16-24 hours. If needed, before spreading the cells, 50µl of X-gal solution (40mg/ml) was spread on the surface of LB plate.

DNA Manipulations

Cloning

Restriction enzyme digestion were carried out by mixing 1-5 µg of DNA with 10-50 Units of restriction enzyme in the appropriate buffer. The total volume for each restriction digest reaction varied from 50 µl to 200 µl. After digestion, 1/20-1/10 of total digested DNA was analyzed by agarose gel electrophoresis, while the remaining was cleaned up by phenol-chloroform extraction and ethanol precipitation.

After digest, if needed, Klenow fragment of *E. coli* DNA polymerase I was used to convert the sticky ends to blunt ends. In a reaction mixture, 5 µg of digested DNA was mixed with Klenow buffer and 1 unit of Klenow fragment of DNA polymerase I in a final

volume of 40 µl. The reaction mixture was incubated at 37 ℃ for 15 minutes before phenol-chloroform-isoamyl alcohol extraction and ethanol precipitation.

Ligation was carried out in 10-20 µl volume by mixing ligase buffer, T_4 DNA ligase, vector DNA and insert DNA (in the ratio of 1:3). The ligation mixture was then incubated at 16 ℃ overnight and 4 ℃ for one extra day before transformation.

Agarose Gel Electrophoresis

DNA samples were analyzed by separation on horizontal agarose gels at room temperature. Agarose gels were made by mixing 1/2X TBE (45 mM Tris-borate, and 1 mM EDTA, pH=8.3) with 0.8 % (w/v) of agarose. DNA samples to be analyzed were mixed with 10 % (v/v) loading buffer [5 % (v/v) glycerol, 3 mM EDTA (pH=8.0), bromophenol blue (0.04 %) and xylene cyanol (0.04 %)] before loading on the gel. The gels were run at 5 volts/cm constant electric current until the forward dye reached 1 cm away from the bottom. After electrophoresis, the gel was soaked in distilled containing ethidium-bromide (1 µg/ml) for 10 minutes to stain DNA and in distilled water for 10 minutes for destaining. The gel images were photographed using an Alpha Imager (Alpha Innotech Corporation). Molecular weight standards (1 kb ladder from BRL) were run along for size reference.

Preparative mini-gels were used to purify DNA fragments of specific sizes. In this case, agarose gels were made with ultra-pure agarose (Bio-Rad). After electrophoresis,

staining and destaining were carried out as above, and a slice of gel slice containing the DNA was electroeluted and purified according to Sambrook [89].

Conjugation

Conjugation between pneumococcal donors and recipients was performed according to the method described by Smith and Guild [90] with minor modifications. Briefly, both donor and recipient cells were grown to an $OD_{550nm} \approx 0.2$ in DIFCo CATPG. The donor and recipient cells were mixed in ratio of 1:10. $MgSO_4$, BSA, and DNase I were added to final concentrations of 10 mM, 2 mg/ml and 5 mg/ml, respectively. Altogether 1 X 10^9 CFU cells were then filtered onto a nitrocellulose filter (Millipore: 13mm diameter and 45 μm pore size). The filter was placed with cell side down on Difco CATPG agar with additional 10 mM $MgSO_4$, 2 mg/ml BSA, 1 mM $CaCl_2$ and 100 μg/ml of DNase I, and agar with the same components was overlaid on top. After the top agar solidified, the plate was moved to a 37℃ incubator and incubated for 4 hours. Then the filter was cut out with both layers of agar and transferred into 3 ml of resuspension broth (CATPG with 10 mM $MgSO_4$, 2 mg/ml BSA, 100 μl/ml DNase I and 10 % glycerol). The cells from the filter were harvested by votexing vigorously, and the culture then stored at -80 ℃ until plating.

Southern Hybridization

Blotting

DNA samples for blotting were firstly digested with appropriate restriction enzymes, and fragments separated on 0.8 % agarose gels by electrophoresis. The gel was stained, destained, and placed under UV light for 30 seconds to break the large fragments of DNA into smaller fragments to increase blotting efficiency. The gel was then soaked in 300 ml denaturation solution (0.4 M NaOH and 0.6 M NaCl) for 30 minutes. After denaturation, the gel was soaked into 300 ml neutralization solution (0.5M Tris-HCl and 1.5M NaCl) for 30 minutes. After neutralization, in a big glass tray, enough 10X SSC, was added to soak a pair of sponges. On top of the sponges, 3 layers of filter paper, the gel containing the DNA for blotting, a pre-cut nylon membrane (GeneScreen Plus, NEN), a second layer of three filter papers, 3 inches of paper towels, and finally a 1 kg weight were overlaid in order. In this setup, DNA was transferred to the nylon membrane successfully through capillary action during overnight incubation.

Pre-hybridization

After blotting, the nylon membrane was transferred to a hybridization tube with 10 ml of pre-hybridization solution (1 g dextran sulfate, 0.58 g NaCl, 1 ml 10 % SDS and 8 ml ddH_2O). The hybridization tube was kept at 65 ℃, rotating overnight.

Nick Translation

Probe DNA was nick-translated as follows, in a 50 µl mixture: 0.5 µl of DNase I (0.5 µg/ml), 5 µl of 10X dNTP without dATP (5 µM for each of dCTP, dGTP and dTTP), 5 µl α-^{32}P- dATP (10 mCi/ml), and 0.5 µl of *E.coli* DNA polymerase I (9000 U/ml). The mixture was kept at 16 ℃ for 2 hours. The reaction was terminated by addition of 25 µl of 0.5 M EDTA (pH=8.0), 25 µl of salmon sperm DNA (2 mg/ml), 50 µl of 7.5 M ammonium acetate, and 50 µl of TE. The DNA was then precipitated by addition of another 400 µl of 95 % ethanol, kept at -20 ℃ for 15 minutes. The DNA was pelleted, dried, and resuspended in 600 µl of TE. To determine the efficiency of isotope incorporation, 1 µl of labeled DNA was mixed with 5ml of liquid scintillation cocktail (Beckman) and the radioactivity was counted in a LS6000SC liquid scintillation counter (Beckman).

Hybridization

Labeled probe DNA ($2*10^6$ CPM) was added to a screw-cap tube with 10 ml hybridization solution [100 µl of 20X SSC, 400µl of salmon sperm DNA (2 mg/ml) in 10 ml]. After boiling for 10 minutes, the screw-cap tube was immediately transferred to ice bath for 3 minutes. Hybridization was carried out by addition of 10 ml of hybridization solution into the hybridization tube containing the membrane. The tube was incubated at 65 ℃, rotating for 18 hours. After discarding hybridization solution, the membrane in the hybridization tube was washed twice with 2X SSPE [17.53 % (w/v) NaCl, 2.76 % (w/v)

TABLE IV. Oligonucleotides used in this study*

647 Em-F	5'-CGATCCTAATAAATTTAAATATCAAAAAGAAGAT
647 Em-R	5'-TCCTTTAACTCTGGCAACCCTCAAAATT
G54-1	5'-TTGAGTCAACCATTAGAAGAAATTCCTC
G54 ORF1-F	5'-ATGGAAGCCATTTATCAACGTGATTCGGAT
MalR-F	5'-gggaattcATTTGGCCAAAATATGACAACC
MalR-R	5'-cgaattcTCTTATTTTTCGATAATTTTGTG
Pm-Mal3	5'-GCAGAATTCAAGTTTTATTGATAAGGAAAC
Pm-Mal4	5'-CGCGGATCCATCTCTAGAGTATTTTGCAGACGCAAACG
pEVP3-F	5'-CTTCCACAGTAGTTCACCACCT
pEVP3-R	5'-ACCCGGGGAGCTCGAATTCTA
VK107	5'-GATATTTAAATTTATTAGGATCGCTCCAATCCTAC -CAAATATTCC
VK109	5'-GCGAGGCAATAATACATTCGGTG
VK110	5'-ATCAATAAAACTTGAATTCTGCTGAAAAAAAA -TTAACTAGGAAAGAAG
VK115	5'-ATTCGGCGGCTCAATCGGGG
VK116	5'-GATACCACCAGGAAGGGCAATAC

*Lower case letters indicate the extra bases added to create the restriction endonuclease sites.

NaH_2PO_4 and 0.74 % (w/v) EDTA (pH=8.0)], for 15 minutes at room temperature, twice with 2X SSPE containing 2% SDS for 45 minutes at 65 ℃, twice in 0.1X SSPE for 30 minutes at room temperature, and once in 3 mM Tris base (unbuffered) solution for 15 min at room temperature. The membrane was dried in a 37 ℃ oven and exposed to X-ray film (Kodak X-Omat AR) at -80 ℃. The exposed film was developed in the Core Facility at Oklahoma State University.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed in an Eppendorf Mastercycler Personal thermal cycler. DNA polymerase used in PCR was deep vent polymerase (New England Biolabs). All the oligonucleotides used for PCR are listed in Table IV. In each cycle of the PCR reaction, 94℃ was used to denature DNA. The annealing temperature in reactions was at least 5 ℃ lower than the Tm of the primer with the lowest Tm. A ramp temperature of 0.3 ℃/minute was applied during annealing. The polymerization temperature was 72 ℃. About 25~30 cycles were run before samples were loaded on preparative gel to purify the amplified DNA.

Virulence assays

For animal model systems, 6 week old female Balb/c mice (Charles River Laboratories, Massachusetts), weighing 25-30 g were used. Bacteria used for infection

were pre-grown to a density of OD_{550}~0.1. The cells were pelleted, resuspended in the same volume of phosphate buffered saline (8 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, and 0.92 g NaH_2PO_4 in 1 liter of distilled water) with 10 % glycerol, and stored in -80 ℃. The cells were thawed on ice and, if needed, they were diluted in PBS and used for infections.

Animal experiments were performed in accordance with the guidelines of Oklahoma State University Institutional Animal Care and Use Committee. For septicemia infection, the pre-stored cultures were diluted to about 1000 cfu/ml and 200 µl injected intraperitoneally. For intranasal infection, mice were lightly anaesthetized with isoflurane (Abbott Laboratories) inhalation and inoculated with $5X10^5$ CFU in 50 µl. Infected mice were monitored for at least 7 days for signs of morbidity and mortality, and in the end, the survivors were euthanized by CO_2 overdose.

Competition experiment

In vivo intranasal competition experiments were performed by inoculation of anaesthetized mice with a bacterial suspension consisting of a 1:1 mixture of the two differentially genetically marked strains (*nov* vs *str*) at a density of 10^6 cfu. Starting at 12 hours post infection, samples were collected by instilling one nostril with 400 µl of sterile PBS and recovering about 200 µl effluent through the other nostril. 100 µl of effluent was mixed with 1.9 ml CAT broth with 10% glycerol and stored at -80 ℃ until plating. For intraperitoneal competition experiments, a 200 µl bacterial suspension containing approximately 1-2 X 10^4 CFU of each bacterium in a 1:1 mixture was injected into mice.

At 24 hours post infection, 5 μl of blood was collected by retro orbital puncture and mixed with 2 ml CAT broth containing 10 % glycerol and stored at -80 ℃ until plating. For plating of nasal washings and cultures from blood, gentamycin (4 μg/ml) was included in all the plates in addition to other antibiotic selections.

Competition experiments *in vitro* were preformed by co-culture of two strains each at about the same density. At different time points, part of the mixture was diluted and plated out under appropriate antibiotic selection to distinguish the viable count of each strain.

Transcriptional Gene Fusion

To determine the transcriptional profile of a gene of interest, gene fusions involving *lacZ* were created as follows. Firstly, a fragment of DNA totally internal to the gene of interest was amplified by PCR using appropriately designed primers. The amplified DNA was then cloned into the S*mal*I site of pEVP3. The recombined plasmid was sequenced by using primers flanking the insertion site to confirm that *lacZ* in the recombined plasmid was in the same orientation as the transcription of the gene of interest. Finally, the recombined plasmid, without linearization, was used as donor DNA to transform a Cm^s pneumococcal strain. The resulting Cm^r transformants were expected to have the directing DNA duplicated flanking the inserted plasmid.

β- Galactosidase assay

These assays were carried out as previously reported [78] with slight modifications. Pneumococcal cells were grown to an optical density as required by each specific experiment. To initiate lysis of the cells, 20 µl of lysis solution [1 0% (v/v) triton with 0.04 % DOC (w/v) in distilled water] was added to each culture. The lysis mixture was incubated at 37 ℃, until the solution became clear. To start the β-galactosidase reaction, 0.5 ml lysate, 0.2 ml of ONPG (10 mg/ml) and 0.5 ml of Z buffer (1.61 g $Na_2HPO_4 \cdot 7H_2O$, 0.55 g $NaH_2PO_4 \cdot H_2O$, 0.075 g KCl, 0.025 g $MgSO_4 \cdot 7H_2O$ and 0.27 ml β-mercaptoethanol in 100 ml distilled water) was mixed into a 1 ml disposable cuvette. The cuvettee was incubated at room temperature until a yellow color was developed. To stop the reaction, 0.5 ml Na_2CO_3 (0.5 M) was added to the reaction mixture and, the optical density at 420nm was measured in a spectrophotometer (Spectronic 1001, Milton Roy Co.). Miller units were calculated according to the formula: Miller unit= $1000*OD_{420}$/time of reaction (minute)/volume of lysate used in reaction (ml)/OD_{550} (cell density)

Hydrogen peroxide susceptibility assays

These assays were carried out as described [15]. Pneumococcal cultures were grown until the OD_{550} of the cultures reached precisely 0.1. The culture was then immediately diluted 1000-fold. The assay was started by addition of H_2O_2 to 1ml of diluted culture to make a final concentration of 10 mM. Another 1 ml of diluted culture

without addition of H_2O_2 was used as a control. Both tubes were kept at 37 ℃ for 30 minutes and then transferred to an ice-bath until plating. Appropriate dilutions were made before being mixed with agar and plated out on top of a CATPG base layer. Finally 10 ml of CATPG-agar with streptomycin (200 μg/ml) was overlaid on top of the cell layer. The percent of survival rate was calculated by dividing the number of cells surviving after exposure to H_2O_2 by the number of cells surviving in the control without exposure to H_2O_2. Similarly, to determine sensitivity of pneumococcus in other oxidative regents, pncumococcal cultures were grown and diluted as described above. Then methyl viologen or tert-butyl hydroperoxide were supplemented to final concentrations of 30 mM and 40 mM, respectively. Survival rates were calculated in the same way as mentioned above.

Hydrogen peroxide production assay

Assays were carried out as described by Pericone et al[15]. Pneumococcal cultures were grown until the OD_{550} reached 0.2. The cells were then pelleted and resuspended in twice the volume of fresh medium. Cell density was determined by measurement of OD_{550}. The cells were incubated in the desired condition (static or aerobic) for 1 hour. Cells were then pelleted again, and the supernatant was saved to determine the concentration of H_2O_2. The assay was performed as follows. In a 1 ml disposable cuvette, 100 μl of the supernatant for aerated culture (900 μl of supernatant for static culture), 100 μl of dyes [3 mg/ml of 2, 2_azinobis (3-ethylbenzthiazolinesulfonic acid) and 0.2 mg.ml horseradish peroxidase] were mixed

with CAT to a final volume to 1 ml. The reaction mixture was incubated at room temperature for 20 minutes and optical density at 560nm then determined by spectrophotometry (Spectronic 1001, Milton Roy Co.). Finally H_2O_2 concentration was calculated by comparison of the OD_{560} to a standard curve generated by using known concentrations of H_2O_2.

Examination of Colony Morphology in Pneumococcal Encapsulated Strains

Examination of colony morphology in pneumococcal encapsulated strains was carried out as described by Weiser et al [91]. Briefly pneumococcal cultures were streaked out on blood agar (CATPG with 2% sheep supplemented) allowing overnight incubation in an anaerobic jar. The next day, the plate was placed under a 100-W halogen substage illuminator to examine colony morphology. Colony of transparent variant appears “smaller, bluish, and more transparent in the center” ("bullseye"), while opaque variant appear “slightly larger, whitish, and more uniform than the other colonies”.

CHAPTER IV

FINDINGS

Features of the methylase/helicase operon in Tn*5252*

Recently, the genomic sequence of a clinical isolate of pneumococcus carrying multiple antibiotic resistance, G54, was published [92]. To determine if antibiotics resistance in G54 was determined by a Tn*5252*-like transposon, we used the sequence of Tn*5252* to search the whole genomic sequence of G54 (http://bioinformatica.cnio.es/data/Spneumo/). A Tn*5252*-like transposon, with almost identical transfer-related genes residing in the termini, was identified in the G54 genomic sequence. Besides the transfer-related genes of the Tn*5252*-like transposon in the G54 genome, an operon was also present in the central region of the element, and the deduced amino acid sequence was almost identical to that from the central segment of Tn*5252*. The operon had a G+C content of 35.1% which was close to the G+C content range of streptococci (35.6%~39.8%). This 7.8 kb region of G54 Tn5252-like element is shown in Fig 2. There are four open reading frames. Upstream of each open reading frame, there was a putative ribosome-binding site. A Gram-positive consensus promoter-like sequence was also observed about 120 bases upstream of the translational start site of ORF1. The sequence between each open reading frame was less than 100 bp and there were no

consensus promoter-like sequence observed upstream of ORF2, ORF3 or ORF4, implying that these four open reading frames are transcribed as one transcriptional unit.

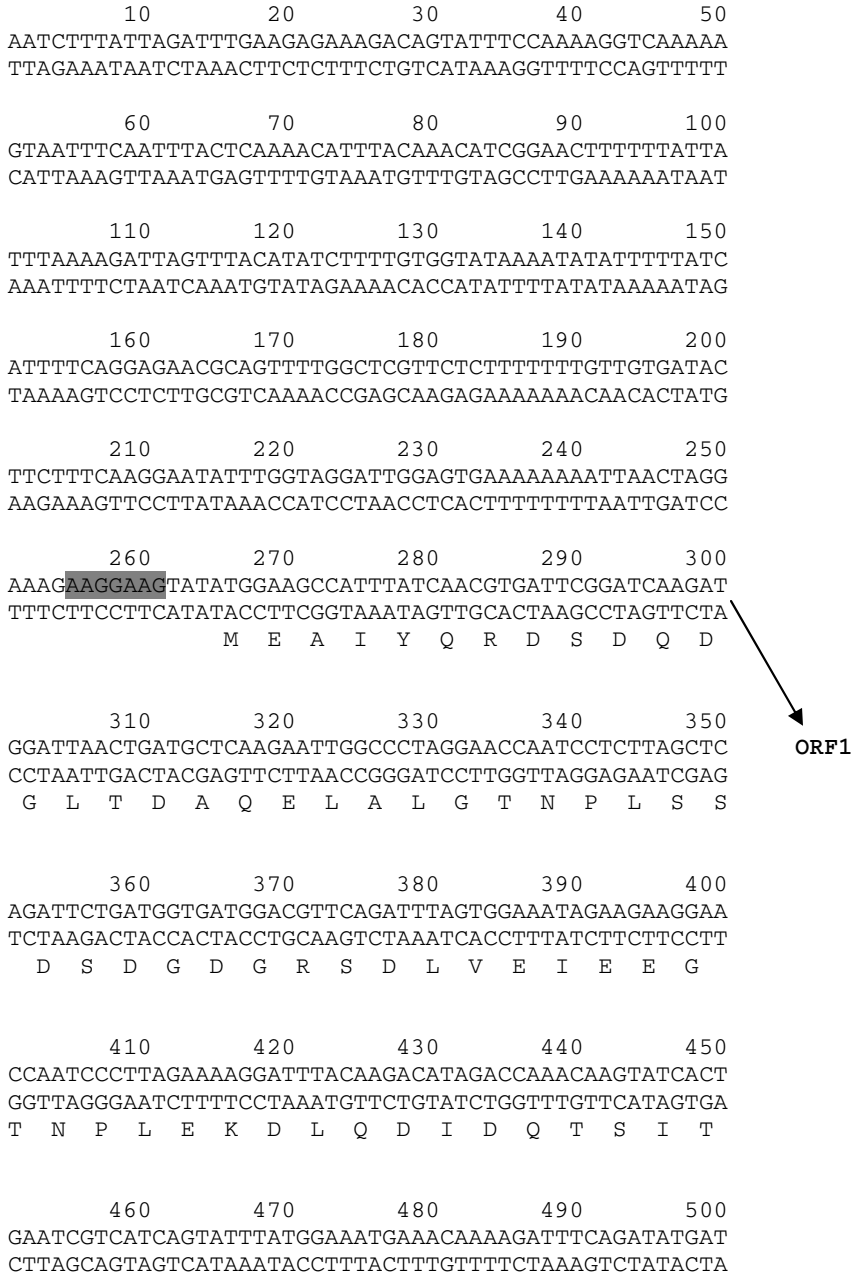
ORF1, ORF3 and ORF4

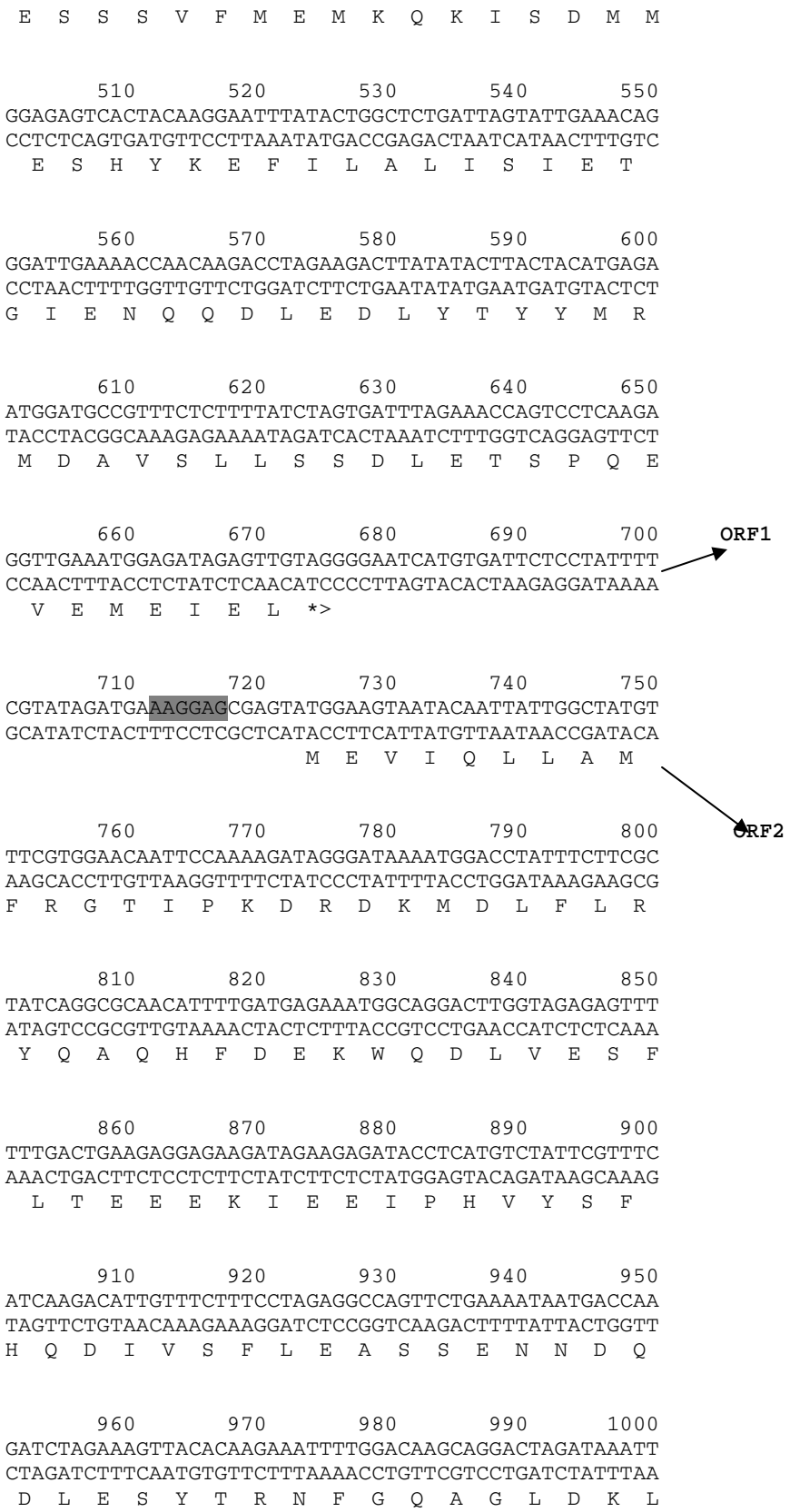
ORF1, ORF3 and ORF4 have the capacity to encode proteins with 136, 94 and 98 amino acids, with molecular masses of 15.4, 11.3 and 11.4 kDa, respectively. Blast search using the deduced amino acid sequences of ORF1, ORF3 and ORF4 revealed the presence of nearly identical sequences in *S. suis* 89/1591 (accession # AAFA02000007.1), *S. agalactiae NEM316* (gbs1355, gbs 1351, and gbs1350 respectively) *and S. agalactiae 2603V/R* (sag1281, sag 1279, and sag1278 respectively). Analysis of the deduced amino acid sequences suggests cytoplasmic localization of the ORF1 and ORF3 proteins, and membrane localization of the ORF4 protein. A Ca^{2+}-binding motif was also present in the protein encoded by ORF1. Proteins encoded by ORF1, ORF3 and ORF4 did not seem to belong to any protein family of known function.

ORF2

Quite different from ORF1, ORF3 and ORF4, ORF2 had the capacity to encode a much larger protein of 2074 amino acids with a molecular weight of 239.8 kDa. Analysis of the deduced amino acid sequence suggests the cytoplasmic localization of this protein. Unlike ORF1, ORF3 and ORF4, the amino acid sequence of ORF2 has significant similarity to sequences from a variety of sources, such as SAG 1280 from *S. agalactiae* 260 V/R, gbs 1352 from *S. agalactiae* NEM 316, EF2307 from *Enterococcus faecalis*

Fig 2. The nucleotide sequence of the 7.8 kb sequence of the helicase operon and the deduced amino acid sequences of ORF1, ORF2, ORF3 and ORF4. The ribosome binding site (RBS) of each of ORF is shown upstream of start codon.




E S S S V F M E M K Q K I S D M M
510 520 530 540 550
GGAGAGTCACTACAAGGAATTTATACTGGCTCTGATTAGTATTGAAACAG
CCTCTCAGTGATGTTCCTTAAATATGACCGAGACTAATCATAACTTTGTC
E S H Y K E F I L A L I S I E T
560 570 580 590 600
GGATTGAAAACCAACAAGACCTAGAAGACTTATATACTTACTACATGAGA
CCTAACTTTTGGTTGTTCTGGATCTTCTGAATATATGAATGATGTACTCT
G I E N Q Q D L E D L Y T Y Y M R
610 620 630 640 650
ATGGATGCCGTTTCTCTTTTATCTAGTGATTTAGAAACCAGTCCTCAAGA
TACCTACGGCAAAGAGAAAATAGATCACTAAATCTTTGGTCAGGAGTTCT
M D A V S L L S S D L E T S P Q E
660 670 680 690 700
GGTTGAAATGGAGATAGAGTTGTAGGGGAATCATGTGATTCTCCTATTTT
CCAACTTTACCTCTATCTCAACATCCCCTTAGTACACTAAGAGGATAAAA
V E M E I E L *>
ORF1
710 720 730 740 750
CGTATAGATGAAAGGAGCGAGTATGGAAGTAATACAATTATTGGCTATGT
GCATATCTACTTTCCTCGCTCATACCTTCATTATGTTAATAACCGATACA
M E V I Q L L A M
760 770 780 790 800
TTCGTGGAACAATTCCAAAAGATAGGGATAAAATGGACCTATTTCTTCGC
AAGCACCTTGTTAAGGTTTTCTATCCCTATTTTACCTGGATAAAGAAGCG
F R G T I P K D R D K M D L F L R
ORF2
810 820 830 840 850
TATCAGGCGCAACATTTTGATGAGAAATGGCAGGACTTGGTAGAGAGTTT
ATAGTCCGCGTTGTAAAACTACTCTTTACCGTCCTGAACCATCTCTCAAA
Y Q A Q H F D E K W Q D L V E S F
860 870 880 890 900
TTTGACTGAAGAGGAGAAGATAGAAGAGATACCTCATGTCTATTCGTTTC
AAACTGACTTCTCCTCTTCTATCTTCTCTATGGAGTACAGATAAGCAAAG
L T E E E K I E E I P H V Y S F
910 920 930 940 950
ATCAAGACATTGTTTCTTTCCTAGAGGCCAGTTCTGAAAATAATGACCAA
TAGTTCTGTAACAAAGAAAGGATCTCCGGTCAAGACTTTTATTACTGGTT
H Q D I V S F L E A S S E N N D Q
960 970 980 990 1000
GATCTAGAAAGTTACACAAGAAATTTTGGACAAGCAGGACTAGATAAATT
CTAGATCTTTCAATGTGTTCTTTAAAACCTGTTCGTCCTGATCTATTTAA
D L E S Y T R N F G Q A G L D K L

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      1010       1020       1030       1040       1050
ATCTCAATTAAGTAATTTTGAGAAAAATTTGGTGCTAAAAGTCGCAAGCT
TAGAGTTAATTCATTAAAACTCTTTTTAAACCACGATTTTCAGCGTTCGA
  S  Q  L  S  N  F  E  K  N  L  V  L  K  V  A  S

      1060       1070       1080       1090       1100
ATAACCTTTCCACTCGATTTTACATCCAATCTGAAAAAGAGAAACTAACA
TATTGGAAAGGTGAGCTAAAATGTAGGTTAGACTTTTTCTCTTTGATTGT
Y  N  L  S  T  R  F  Y  I  Q  S  E  K  E  K  L  T

      1110       1120       1130       1140       1150
CCATTAAGTGAGCTTGTATTTCATCAGAATCAGGATGTTAATTTAGTCAA
GGTAATTCACTCGAACATAAAGTAGTCTTAGTCCTACAATTAAATCAGTT
 P  L  S  E  L  V  F  H  Q  N  Q  D  V  N  L  V  N

      1160       1170       1180       1190       1200
TGTCTATCGGGTTGCGAATAATCTATCTGACCGTATTAGTAGAGATATAG
ACAGATAGCCCAACGCTTATTAGATAGACTGGCATAATCATCTCTATATC
  V  Y  R  V  A  N  N  L  S  D  R  I  S  R  D  I

      1210       1220       1230       1240       1250
AGGAATTTCTTCTAATGGTTGACTCAAAAGAGGTTAAAAAAGAAGTTCCT
TCCTTAAAGAAGATTACCAACTGAGTTTTCTCCAATTTTTTCTTCAAGGA
E  E  F  L  L  M  V  D  S  K  E  V  K  K  E  V  P

      1260       1270       1280       1290       1300
GAGATTCATTTTGAAGAAAAAGAGGGAGATGTTCTAGCCTATTTGGGTTC
CTCTAAGTAAAACTTCTTTTTCTCCCTCTACAAGATCGGATAAACCCAAG
 E  I  H  F  E  E  K  E  G  D  V  L  A  Y  L  G  S

      1310       1320       1330       1340       1350
AGAATTGATGGCTACTTTAGATATCGTTACGGATCTTGTCCATCATGAAG
TCTTAACTACCGATGAAATCTATAGCAATGCCTAGAACAGGTAGTACTTC
  E  L  M  A  T  L  D  I  V  T  D  L  V  H  H  E

      1360       1370       1380       1390       1400
AAAACTACATACAACTCCCACTGACACAAAAGCTGAAGATTATTACTCAT
TTTTGATGTATGTTGAGGGTGACTGTGTTTTCGACTTCTAATAATGAGTA
E  N  Y  I  Q  L  P  L  T  Q  K  L  K  I  I  T  H

      1410       1420       1430       1440       1450
TTTGATGAAGTAAAAGCTAAAAGCGAAAAGTCTAATCAAGTAGAGAAAAT
AAACTACTTCATTTTCGATTTTCGCTTTTCAGATTAGTTCATCTCTTTTA
 F  D  E  V  K  A  K  S  E  K  S  N  Q  V  E  K  I

      1460       1470       1480       1490       1500
TTTATCTCCTTCAAGTGATATTGAACAGGAAACGGAAGAAACTAACTCCT
AAATAGAGGAAGTTCACTATAACTTGTCCTTTGCCTTCTTTGATTGAGGA
  L  S  P  S  S  D  I  E  Q  E  T  E  E  T  N  S

      1510       1520       1530       1540       1550
TTTCTAATGTCGATAAAATTGTAGAAGAAGCTTTGAGGGAATATCCAATC
AAAGATTACAGCTATTTTAACATCTTCTTCGAAACTCCCTTATAGGTTAG
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F  S  N  V  D  K  I  V  E  E  A  L  R  E  Y  P  I

      1560       1570       1580       1590       1600
GGTTCACAAGTAAGTTATAAAGGACAAGTATTTCAGTTGGTTTCGATTGA
CCAAGTGTTCATTCAATATTTCCTGTTCATAAAGTCAACCAAAGCTAACT
 G  S  Q  V  S  Y  K  G  Q  V  F  Q  L  V  S  I  E

      1610       1620       1630       1640       1650
AAATGCGCAGTTAAATGACTTAGTTCGCCTAGAGCTATTCAATGATTCCA
TTTACGCGTCAATTTACTGAATCAAGCGGATCTCGATAAGTTACTAAGGT
  N  A  Q  L  N  D  L  V  R  L  E  L  F  N  D  S

      1660       1670       1680       1690       1700
ACCAGTTATTTGAAGAGAATCCTATCTTATACTTGAACAGTTTGGAAGAG
TGGTCAATAAACTTCTCTTAGGATAGAATATGAACTTGTCAAACCTTCTC
N  Q  L  F  E  E  N  P  I  L  Y  L  N  S  L  E  E

      1710       1720       1730       1740       1750
ATTGAACAAGTATTGTCTCATTTAGAACTTGAAAAAGAAGATTCAGAGAT
TAACTTGTTCATAACAGAGTAAATCTTGAACTTTTTCTTCTAAGTCTCTA
 I  E  Q  V  L  S  H  L  E  L  E  K  E  D  S  E  I

      1760       1770       1780       1790       1800
TGAGATTGATTCATCAAGTGAAAGTCAGGAAATAGATTTGTTTTCATACC
ACTCTAACTAAGTAGTTCACTTTCAGTCCTTTATCTAAACAAAAGTATGG
  E  I  D  S  S  S  E  S  Q  E  I  D  L  F  S  Y

      1810       1820       1830       1840       1850
TGGAAGAAGAAAATGTAAATGAAAAGGATAAGGATAAGGAAACAAAATCT
ACCTTCTTCTTTTACATTTACTTTTCCTATTCCTATTCCTTTGTTTTAGA
L  E  E  E  N  V  N  E  K  D  K  D  K  E  T  K  S

      1860       1870       1880       1890       1900
TTAATTTCAGGTATAGAAGAGACGGATGTCCCTGTTCTAGATTTTGTTTT
AATTAAAGTCCATATCTTCTCTGCCTACAGGGACAAGATCTAAAACAAAA
 L  I  S  G  I  E  E  T  D  V  P  V  L  D  F  V  F

      1910       1920       1930       1940       1950
TCCAGATGATTTAGAGGACTTTTATCCTAAGACAAATCGAGAAAAGATTG
AGGTCTACTAAATCTCCTGAAAATAGGATTCTGTTTAGCTCTTTTCTAAC
  P  D  D  L  E  D  F  Y  P  K  T  N  R  E  K  I

      1960       1970       1980       1990       2000
AAACGAATATCGCCGCAATTGAACTTGTTAAAAGATTAGAAAAAGAGAGA
TTTGCTTATAGCGGCGTTAACTTGAACAATTTTCTAATCTTTTTCTCTCT
E  T  N  I  A  A  I  E  L  V  K  R  L  E  K  E  R

      2010       2020       2030       2040       2050
CGACAAGCGAATCCAGAAGAACAAGAGCTACTCGCCAAGTATGTCGGCTG
GCTGTTCGCTTAGGTCTTCTTGTTCTCGATGAGCGGTTCATACAGCCGAC
 R  Q  A  N  P  E  E  Q  E  L  L  A  K  Y  V  G  W
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      2060       2070       2080       2090       2100
GGGCGGTCTTGCCAATGAATTTTTCGATGAACTCAATCCAAAGTATGAAA
CCCGCCAGAACGGTTACTTAAAAAGCTACTTGAGTTAGGTTTCATACTTT
  G  G  L  A  N  E  F  F  D  E  L  N  P  K  Y  E

      2110       2120       2130       2140       2150
CAGAACGTTTAACTCTTAAGAGCTTAGTAAGTAAATCAGAATACTCGACC
GTCTTGCAAATTGAGAATTCTCGAATCATTCATTTAGTCTTATGAGCTGG
T  E  R  L  T  L  K  S  L  V  S  K  S  E  Y  S  T

      2160       2170       2180       2190       2200
ATAAAACAAAGTTCTCTCACAGCCTATTATACAGACCCAATGATTATTCG
TATTTTGTTTCAAGAGAGTGTCGGATAATATGTCTGGGTTACTAATAAGC
 I  K  Q  S  S  L  T  A  Y  Y  T  D  P  M  I  I  R

      2210       2220       2230       2240       2250
CCAGATTTGGCAAAAATTACTGGATGATGGTTTTGAGGGAGGAAGGATAT
GGTCTAAACCGTTTTTAATGACCTACTACCAAAACTCCCTCCTTCCTATA
  Q  I  W  Q  K  L  L  D  D  G  F  E  G  G  R  I

      2260       2270       2280       2290       2300
TAGATCCTTCTATGGGGACTGGGAACTTCTTTGCGGCGATGCCTAGAAGT
ATCTAGGAAGATACCCCTGACCCTTGAAGAAACGCCGCTACGGATCTTCA
L  D  P  S  M  G  T  G  N  F  F  A  A  M  P  R  S

      2310       2320       2330       2340       2350
ATACGAGAGAAATCAGAACTCTATGGGGTTGAATTAGACAGTGTGACTGG
TATGCTCTCTTTAGTCTTGAGATACCCCAACTTAATCTGTCACACTGACC
 I  R  E  K  S  E  L  Y  G  V  E  L  D  S  V  T  G

      2360       2370       2380       2390       2400
TGCAATCGCAAAACAACTCCACCCCAATACCCATATTGAAGTGCGAGGAT
ACGTTAGCGTTTTGTTGAGGTGGGGTTATGGGTATAACTTCACGCTCCTA
  A  I  A  K  Q  L  H  P  N  T  H  I  E  V  R  G

      2410       2420       2430       2440       2450
TTGAAGAAGTTCCCTATCAAAATAATAGTTTTGATTTAGTCTTAACGAAT
AACTTCTTCAAGGGATAGTTTTATTATCAAAACTAAATCAGAATTGCTTA
F  E  E  V  P  Y  Q  N  N  S  F  D  L  V  L  T  N

      2460       2470       2480       2490       2500
GTTCCTTTTGGAAATTTTCGCATTGCCGATAAAAACTATGATAAACCTTA
CAAGGAAAACCTTTAAAAGCGTAACGGCTATTTTTGATACTATTTGGAAT
 V  P  F  G  N  F  R  I  A  D  K  N  Y  D  K  P  Y

      2510       2520       2530       2540       2550
TATGATTCATGATTACTTTGTCAAACACTCACTTGATTTAGTAAGAGACG
ATACTAAGTACTAATGAAACAGTTTGTGAGTGAACTAAATCATTCTCTGC
  M  I  H  D  Y  F  V  K  H  S  L  D  L  V  R  D

      2560       2570       2580       2590       2600
GAGGACAAGTGTCGATTATCTCATCTATTGGGACAATGGATAAGCGGACA
CTCCTGTTCACAGCTAATAGAGTAGATAACCCTGTTACCTATTCGCCTGT
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G  G  Q  V  S  I  I  S  S  I  G  T  M  D  K  R  T

      2610       2620       2630       2640       2650
GATAATGTCTTACAAGAGATTAAATCCAATACTCATTTTTTAGGGGGAGT
CTATTACAGAATGTTCTCTAATTTAGGTTATGAGTAAAAAATCCCCCTCA
 D  N  V  L  Q  E  I  K  S  N  T  H  F  L  G  G  V

      2660       2670       2680       2690       2700
TCGGTTGCCGGATACGGCTTTTAAAAAGATTGCAGGTACCCGAGTGACCA
AGCCAACGGCCTATGCCGAAAATTTTTCTAACGTCCATGGGCTCACTGGT
  R  L  P  D  T  A  F  K  K  I  A  G  T  R  V  T

      2710       2720       2730       2740       2750
CAGATCTCCTATTCTTTCAAAAGGATCAAGCAAAGAATCTTAATGAGGAG
GTCTAGAGGATAAGAAAGTTTTCCTAGTTCGTTTCTTAGAATTACTCCTC
T  D  L  L  F  F  Q  K  D  Q  A  K  N  L  N  E  E

      2760       2770       2780       2790       2800
GAACTTGTCTTTAGTGGCTCTGTTCCCTTTGAGGAGGATAAGCGTGTCTG
CTTGAACAGAAATCACCGAGACAAGGGAAACTCCTCCTATTCGCACAGAC
 E  L  V  F  S  G  S  V  P  F  E  E  D  K  R  V  W

      2810       2820       2830       2840       2850
GATCAATCCTTATTTTGATGGGAAATACAATACACAAGTTTTGGGTGAAT
CTAGTTAGGAATAAAACTACCCTTTATGTTATGTGTTCAAAACCCACTTA
  I  N  P  Y  F  D  G  K  Y  N  T  Q  V  L  G  E

      2860       2870       2880       2890       2900
ATGAGGTACGTAATTTTAATGGAGGAACCCTCAATGTTAAGGGGGAATCA
TACTCCATGCATTAAAATTACCTCCTTGGGAGTTACAATTCCCCCTTAGT
Y  E  V  R  N  F  N  G  G  T  L  N  V  K  G  E  S

      2910       2920       2930       2940       2950
GAAACATTAGCTACTGACATAATGAAAGCATTAGAGAATGTAGAAGCACC
CTTTGTAATCGATGACTGTATTACTTTCGTAATCTCTTACATCTTCGTGG
 E  T  L  A  T  D  I  M  K  A  L  E  N  V  E  A  P

      2960       2970       2980       2990       3000
TAAACAAATTGACAATTCTTTGAAAGCACCTGTTTTTATCCAAGAAGAAG
ATTTGTTTAACTGTTAAGAAACTTTCGTGGACAAAAATAGGTTCTTCTTC
  K  Q  I  D  N  S  L  K  A  P  V  F  I  Q  E  E

      3010       3020       3030       3040       3050
TGGATAATTCTATCCCAAGTCATATACGTGAGAACTTAGCGCTCTATTCT
ACCTATTAAGATAGGGTTCAGTATATGCACTCTTGAATCGCGAGATAAGA
V  D  N  S  I  P  S  H  I  R  E  N  L  A  L  Y  S

      3060       3070       3080       3090       3100
TTTGGATATGAGGGAAATCAAATTTATTATCGAGATACGCATGGCATTCG
AAACCTATACTCCCTTTAGTTTAAATAATAGCTCTATGCGTACCGTAAGC
 F  G  Y  E  G  N  Q  I  Y  Y  R  D  T  H  G  I  R
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      3110       3120       3130       3140       3150
GAGAAGTTCAAAAGTAGACGAAATTAGTTATTATGTAGATGAGAAGGGAG
CTCTTCAAGTTTTCATCTGCTTTAATCAATAATACATCTACTCTTCCCTC
  R  S  S  K  V  D  E  I  S  Y  Y  V  D  E  K  G

      3160       3170       3180       3190       3200
ATTTTAAAGCTTGGGACAGTTCCTTGTCTGAACATAAAATAGATCGATTC
TAAAATTTCGAACCCTGTCAAGGAACAGACTTGTATTTTATCTAGCTAAG
D  F  K  A  W  D  S  S  L  S  E  H  K  I  D  R  F

      3210       3220       3230       3240       3250
GTGCAACTTCATTTGACAGATGAGGAAGCACTAGATGTATACAAGTCAGA
CACGTTGAAGTAAACTGTCTACTCCTTCGTGATCTACATATGTTCAGTCT
 V  Q  L  H  L  T  D  E  E  A  L  D  V  Y  K  S  E

      3260       3270       3280       3290       3300
AGAAGCGAGTAAAAGAGGGAAATATAAGGGCTTGTTCAAAAAAACGGTCT
TCTTCGCTCATTTTCTCCCTTTATATTCCCGAACAAGTTTTTTTGCCAGA
  E  A  S  K  R  G  K  Y  K  G  L  F  K  K  T  V

      3310       3320       3330       3340       3350
TTTATGAAAGCCCCTTATCGGATAAGGATATTAGTCGCATTAAGGGCATG
AAATACTTTCGGGGAATAGCCTATTCCTATAATCAGCGTAATTCCCGTAC
F  Y  E  S  P  L  S  D  K  D  I  S  R  I  K  G  M

      3360       3370       3380       3390       3400
GTTGATTTGAGAGAGACCTATCAAGCCTTAATTGAAATTCAACGCCATCC
CAACTAAACTCTCTCTGGATAGTTCGGAATTAACTTTAAGTTGCGGTAGG
 V  D  L  R  E  T  Y  Q  A  L  I  E  I  Q  R  H  P

      3410       3420       3430       3440       3450
AGATTATAGTCGGACAGATTTTCAGGTATTACTTAGTAAACTCAATCGTG
TCTAATATCAGCCTGTCTAAAAGTCCATAATGAATCATTTGAGTTAGCAC
  D  Y  S  R  T  D  F  Q  V  L  L  S  K  L  N  R

      3460       3470       3480       3490       3500
ACTATGATCGGTTTGTAAGCCAATTTGGATACTTGAATGTCTCAGTCAAT
TGATACTAGCCAAACATTCGGTTAAACCTATGAACTTACAGAGTCAGTTA
D  Y  D  R  F  V  S  Q  F  G  Y  L  N  V  S  V  N

      3510       3520       3530       3540       3550
CGAAACTTATTTGATAGTGACGATAAGTATTCTTTACTAGCAAGTTTAGA
GCTTTGAATAAACTATCACTGCTATTCATAAGAAATGATCGTTCAAATCT
 R  N  L  F  D  S  D  D  K  Y  S  L  L  A  S  L  E

      3560       3570       3580       3590       3600
AGATGAATACATTGATTCTAAAGATCAGAAAGTAAAATATAAAAAATCTT
TCTACTTATGTAACTAAGATTTCTAGTCTTTCATTTTATATTTTTTAGAA
  D  E  Y  I  D  S  K  D  Q  K  V  K  Y  K  K  S

      3610       3620       3630       3640       3650
TAGCCTTTGAGAAAGCATTGGTTAGGCCGGAGAGAGTGATTACAAGAGTT
ATCGGAAACTCTTTCGTAACCAATCCGGCCTCTCTCACTAATGTTCTCAA
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L  A  F  E  K  A  L  V  R  P  E  R  V  I  T  R  V

      3660       3670       3680       3690       3700
TCAACGGCTTTAGATGCCTTAAACTCCAGTTTATCAGATGGTAGAGGGGT
AGTTGCCGAAATCTACGGAATTTGAGGTCAAATAGTCTACCATCTCCCCA
 S  T  A  L  D  A  L  N  S  S  L  S  D  G  R  G  V

      3710       3720       3730       3740       3750
TGATATAGACTTTATGGTATCAATTTACCCTGAACATAGCCAAGCAGCTA
ACTATATCTGAAATACCATAGTTAAATGGGACTTGTATCGGTTCGTCGAT
  D  I  D  F  M  V  S  I  Y  P  E  H  S  Q  A  A

      3760       3770       3780       3790       3800
TTTTAGATGAGTTGGGTGACCAGATCTTAATGGATCCAGAAAGCTATTTA
AAAATCTACTCAACCCACTGGTCTAGAATTACCTAGGTCTTTCGATAAAT
I  L  D  E  L  G  D  Q  I  L  M  D  P  E  S  Y  L

      3810       3820       3830       3840       3850
AGAGGGGAAAGAAATTATCTTTCTAAGAACCAGTTTTTGTCAGGAGATAT
TCTCCCCTTTCTTTAATAGAAAGATTCTTGGTCAAAAACAGTCCTCTATA
 R  G  E  R  N  Y  L  S  K  N  Q  F  L  S  G  D  I

      3860       3870       3880       3890       3900
TCTCAACAAGATAGAAGTAGTTCAACTATTAGTAGAGGAAAACAACCAAG
AGAGTTGTTCTATCTTCATCAAGTTGATAATCATCTCCTTTTGTTGGTTC
  L  N  K  I  E  V  V  Q  L  L  V  E  E  N  N  Q

      3910       3920       3930       3940       3950
AATGTGATTGGACTCATGCGTTAGATTTGTTAGAATCTGTTCGCCCTCCA
TTACACTAACCTGAGTACGCAATCTAAACAATCTTAGACAAGCGGGAGGT
E  C  D  W  T  H  A  L  D  L  L  E  S  V  R  P  P

      3960       3970       3980       3990       4000
AGGATTCATCTGGCAGATATTGAGTTTAAAATAGGGTCACGTTGGATTCC
TCCTAAGTAGACCGTCTATAACTCAAATTTTATCCCAGTGCAACCTAAGG
 R  I  H  L  A  D  I  E  F  K  I  G  S  R  W  I  P

      4010       4020       4030       4040       4050
TCAATCCGTTTATGGTAAATTTGCCTTTGAATGTTTTACCAACCGTGAAT
AGTTAGGCAAATACCATTTAAACGGAAACTTACAAAATGGTTGGCACTTA
  Q  S  V  Y  G  K  F  A  F  E  C  F  T  N  R  E

      4060       4070       4080       4090       4100
TTGAATTGTCTTCGCCTGATGTTGAACAAGTCATTGAAGTGAATCCTGTC
AACTTAACAGAAGCGGACTACAACTTGTTCAGTAACTTCACTTAGGACAG
F  E  L  S  S  P  D  V  E  Q  V  I  E  V  N  P  V

      4110       4120       4130       4140       4150
GATGGGCAGGTTCATTTAAGGACACCATTTGCTTATCGCTATCCAAGTGC
CTACCCGTCCAAGTAAATTCCTGTGGTAAACGAATAGCGATAGGTTCACG
 D  G  Q  V  H  L  R  T  P  F  A  Y  R  Y  P  S  A

      4160       4170       4180       4190       4200
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CAAAGATAGTAGTCTTGGAGTCAGTGGCTCACGTTATGATACAGGAAGAA
GTTTCTATCATCAGAACCTCAGTCACCGAGTGCAATACTATGTCCTTCTT
  K  D  S  S  L  G  V  S  G  S  R  Y  D  T  G  R

      4210       4220       4230       4240       4250
AGATTTTTGAGAATTTACTTAATTCGAACCAACCGACTATTACTATGACT
TCTAAAAAACTCTTAAATGAATTAAGCTTGGTTGGCTGATAATGATACTGA
K  I  F  E  N  L  L  N  S  N  Q  P  T  I  T  M  T

      4260       4270       4280       4290       4300
GTTACGGAAGGGGAAAAGAAAAAGACCATCACAGATTTGGAAAAAACCTC
CAATGCCTTCCCCTTTTCTTTTTCTGGTAGTGTCTAAACCTTTTTTGGAG
 V  T  E  G  E  K  K  K  T  I  T  D  L  E  K  T  S

      4310       4320       4330       4340       4350
TGTTCTAAGAGCAAAAGAGCAGCATTTACAAGAACTCTTTCAAGACTTTG
ACAAGATTCTCGTTTTCTCGTCGTAAATGTTCTTGAGAAAGTTCTGAAAC
  V  L  R  A  K  E  Q  H  L  Q  E  L  F  Q  D  F

      4360       4370       4380       4390       4400
TCTCACGGTATCCAGAAGTTCAACAAGTCATTGAAGAAAGTTATAATCGT
AGAGTGCCATAGGTCTTCAAGTTGTTCAGTAACTTCTTTCAATATTAGCA
V  S  R  Y  P  E  V  Q  Q  V  I  E  E  S  Y  N  R

      4410       4420       4430       4440       4450
CTTTATAATCGAACGGTTAGTCGAGAGTATGACGGTAGCCATTTAGTCAT
GAAATATTAGCTTGCCAATCAGCTCTCATACTGCCATCGGTAAATCAGTA
 L  Y  N  R  T  V  S  R  E  Y  D  G  S  H  L  V  I

      4460       4470       4480       4490       4500
TGATGGCTTGGCACAAAACATCAGTCTTCGTCCTCATCAAGAGAATGCCA
ACTACCGAACCGTGTTTTGTAGTCAGAAGCAGGAGTAGTTCTCTTACGGT
  D  G  L  A  Q  N  I  S  L  R  P  H  Q  E  N  A

      4510       4520       4530       4540       4550
TTCAAAGAATTGTAGAAGAAAAAAGAGCCTTGTTAGCTCATGAGGTAGGT
AAGTTTCTTAACATCTTCTTTTTTCTCGGAACAATCGAGTACTCCATCCA
I  Q  R  I  V  E  E  K  R  A  L  L  A  H  E  V  G

      4560       4570       4580       4590       4600
TCAGGAAAGACCTTGACCATGCTTGGTGCTGGCTTTAAATTAAAGGAGTT
AGTCCTTTCTGGAACTGGTACGAACCACGACCGAAATTTAATTTCCTCAA
 S  G  K  T  L  T  M  L  G  A  G  F  K  L  K  E  L

      4610       4620       4630       4640       4650
GGGGATGGTTCATAAGCCCTTGTATGTGGTGCCCTCTAGTTTGTCTGCTC
CCCCTACCAAGTATTCGGGAACATACACCACGGGAGATCAAACAGACGAG
  G  M  V  H  K  P  L  Y  V  V  P  S  S  L  S  A

      4660       4670       4680       4690       4700
AGTTTGGCCAAGAAATCATGAAATTTTTCCCTACTAAAAAAGTCTTTGTG
TCAAACCGGTTCTTTAGTACTTTAAAAAGGGATGATTTTTTCAGAAACAC
Q  F  G  Q  E  I  M  K  F  F  P  T  K  K  V  F  V
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      4710      4720      4730      4740      4750
ACCACTAAGAAAGATTTTGTGAAGGCAAGAAGAAAACAGTTTGTGTCACG
TGGTGATTCTTTCTAAAACACTTCCGTTCTTCTTTTGTCAAACACAGTGC
  T   T   K   K   D   F   V   K   A   R   R   K   Q   F   V   S   R

      4760      4770      4780      4790      4800
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ATAATAATGTCCTCTAATGCTACGGTAACAGTAACCCCTAAGAGTTAAAC
    I   I   T   G   D   Y   D   A   I   V   I   G   D   S   Q   F

      4810      4820      4830      4840      4850
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TTTTTTAGGGACAGTCATTCCTTTCTGTCTACTTAATATAGCTCCTATTT
E   K   I   P   V   S   K   E   R   Q   M   N   Y   I   E   D   K

      4860      4870      4880      4890      4900
CTCAATGAACTACGAGAGATTAAAACACATTCTGAAAATAAGTACACCGT
GAGTTACTTGATGCTCTCTAATTTTGTGTAAGACTTTTATTCATGTGGCA
  L   N   E   L   R   E   I   K   T   H   S   E   N   K   Y   T   V

      4910      4920      4930      4940      4950
TAAAGAAGCAGAGCAATCAATAAGTGGTCTTGAGAAACAATTGGAAGAAC
ATTTCTTCGTCTCGTTAGTTATTCACCAGAACTCTTTGTTAACCTTCTTG
    K   E   A   E   Q   S   I   S   G   L   E   K   Q   L   E   E

      4960      4970      4980      4990      5000
TCCAACGCTTTAATCGTGATAGTTTTATTGATTTTGAGAACTTAGGAATT
AGGTTGCGAAATTAGCACTATCAAAATAACTAAAACTCTTGAATCCTTAA
L   Q   R   F   N   R   D   S   F   I   D   F   E   N   L   G   I

      5010      5020      5030      5040      5050
GATTTTCTCTTTGTGGATGAAGCACATCACTTTAAAAATATACGTCCAAT
CTAAAAGAGAAACACCTACTTCGTGTAGTGAAATTTTTATATGCAGGTTA
  D   F   L   F   V   D   E   A   H   H   F   K   N   I   R   P   I

      5060      5070      5080      5090      5100
TACTGGACTTGGAAATGTAGCAGGGATTACCAATACAACGTCTAAGAAGA
ATGACCTGAACCTTTACATCGTCCCTAATGGTTATGTTGCAGATTCTTCT
    T   G   L   G   N   V   A   G   I   T   N   T   T   S   K   K

      5110      5120      5130      5140      5150
ATGTGGATATGGAAATGAAGGTTCGACAGATTCAGGAAGAACATGATTTT
TACACCTATACCTTTACTTCCAAGCTGTCTAAGTCCTTCTTGTACTAAAA
N   V   D   M   E   M   K   V   R   Q   I   Q   E   E   H   D   F

      5160      5170      5180      5190      5200
AAAAATATTGTCTTTGCGACAGGAACACCTGTTTCAAATTCAATTAGTGA
TTTTTATAACAGAAACGCTGTCCTTGTGGACAAAGTTTAAGTTAATCACT
  K   N   I   V   F   A   T   G   T   P   V   S   N   S   I   S   E

      5210      5220      5230      5240      5250
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CGACATATGATACTACTTGATGTAAGTTGGCCTATAGAATTTTGCGATAG
  L  Y  T  M  M  N  Y  I  Q  P  D  I  L  K  R  Y

      5260      5270      5280      5290      5300
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TTCAACTAATAAAACTGAGAACCCATCCACGAAAACCTCTTTAAGTTTTG
Q  V  D  Y  F  D  S  W  V  G  A  F  G  E  I  Q  N

      5310      5320      5330      5340      5350
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AGATACCTTAATCGAGGATGTCCCCTATTCATGGTCGGATTCTTTGCTAA
 S  M  E  L  A  P  T  G  D  K  Y  Q  P  K  K  R  F

      5360      5370      5380      5390      5400
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ATTTTTCAAACAGTTAGATGGACTCAACTACTTTTAGATATTTCTTTGTC
  K  K  F  V  N  L  P  E  L  M  K  I  Y  K  E  T

      5410      5420      5430      5440      5450
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GGCTGTAAGTTTGTGTTCTATACAACCTAAATGGACATGGTCTTCGGGTA
A  D  I  Q  T  Q  D  M  L  D  L  P  V  P  E  A  H

      5460      5470      5480      5490      5500
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TAATAGGGATAACTCTCACTCAATTGACTTTTGGTCTTTGAGATAGATCT
 I  I  P  I  E  S  E  L  T  E  N  Q  K  L  Y  L  E

      5510      5520      5530      5540      5550
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TCTTAACCAATACTCTAGTCTACGCCAGTTTACACCTTGTCAACTAGGTT
  E  L  V  M  R  S  D  A  V  K  C  G  T  V  D  P

      5560      5570      5580      5590      5600
GTCAGGATAACATGTTAAAAATTACGGGTGAGGCACGAAAATTAGCTATT
CAGTCCTATTGTACAATTTTTAATGCCCACTCCGTGCTTTTAATCGATAA
S  Q  D  N  M  L  K  I  T  G  E  A  R  K  L  A  I

      5610      5620      5630      5640      5650
GATATGCGTTTATTGGACTCTAGTTATAGTCTAGCAGACAATCATAAACT
CTATACGCAAATAACCTGAGATCAATATCAGATCGTCTGTTAGTATTTGA
 D  M  R  L  L  D  S  S  Y  S  L  A  D  N  H  K  L

      5660      5670      5680      5690      5700
GCTTCAGGTAGTGGATAATGTTGAAAGAATTTATCGTGAGGGAATGGAAA
CGAAGTCCATCACCTATTACAACTTTCTTAAATAGCACTCCCTTACCTTT
  L  Q  V  V  D  N  V  E  R  I  Y  R  E  G  M  E

      5710      5720      5730      5740      5750
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TATTCCGATGAGTCTACTAAAAAAGTCTATAACCGTGTGGATTCTTTTTT
N  K  A  T  Q  M  I  F  S  D  I  G  T  P  K  K  K
```

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      5760       5770       5780       5790       5800
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CTATTACCGAAACTACAAATAAGACTCTAATTCCGAAATAATCAACTATC
 D  N  G  F  D  V  Y  S  E  I  K  A  L  L  V  D  R

      5810       5820       5830       5840       5850
AGGAATCCCTAGTATGGAAATTGCCTTTGTACATGATGCCAATAGTGATG
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  G  I  P  S  M  E  I  A  F  V  H  D  A  N  S  D

      5860       5870       5880       5890       5900
AAAAGAAGAATAGCTTGTCTCGAAAGGTTAATGCAGGAGAGGTTCGGGTT
TTTTCTTCTTATCGAACAGAGCTTTCCAATTACGTCCTCTCCAAGCCCAA
E  K  K  N  S  L  S  R  K  V  N  A  G  E  V  R  V

      5910       5920       5930       5940       5950
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GAAGAACGGAGTTGACTTTTTCCTCCTTGTCCAAATTTACAAGTCTCGTT
 L  L  A  S  T  E  K  G  G  T  G  L  N  V  Q  S  K

      5960       5970       5980       5990       6000
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CTACTTTCGTCAAGTGGTAGACCTACATGGCACCTCTGGTTCACTGTAAG
  M  K  A  V  H  H  L  D  V  P  W  R  P  S  D  I

      6010       6020       6030       6040       6050
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TCGTTGCATTACCTGCATAATAGGCTGTCCCTTTACTTTTGTTCCTTCAC
Q  Q  R  N  G  R  I  I  R  Q  G  N  E  N  K  E  V

      6060       6070       6080       6090       6100
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CTATAAATGGTGATATAATGGTTTCCAAGCAAACTATTAATAGATACCCG
 D  I  Y  H  Y  I  T  K  G  S  F  D  N  Y  L  W  A

      6110       6120       6130       6140       6150
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TTGAGTCCTCTTGTTTGAGGCAATATAATTCGTCTAATACTGAAGATTCC
  T  Q  E  N  K  L  R  Y  I  K  Q  I  M  T  S  K

      6160       6170       6180       6190       6200
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TCGGCTAAGCACGACGCCTTCTATAACTACTTGTCTGATACTGTCGAAGA
E  P  I  R  A  A  E  D  I  D  E  Q  T  M  T  A  S

      6210       6220       6230       6240       6250
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CTAAAATTCCGTGATCGTTGTCCATTAGGAATAGAGTTTATATTCTACCT
 D  F  K  A  L  A  T  G  N  P  Y  L  K  Y  K  M  E

      6260       6270       6280       6290       6300
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```

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  L  E  N  D  L  T  L  L  E  N  Q  R  R  A  F  Q

      6310       6320       6330       6340       6350
GCAGCAAGGATCACTATCGTCATACAATCTCTTACTGTGAAGAAAATATG
CGTCGTTCCTAGTGATAGCAGTATGTTAGAGAATGACACTTCTTTTATAC
R  S  K  D  H  Y  R  H  T  I  S  Y  C  E  E  N  M

      6360       6370       6380       6390       6400
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GGGTAAGAACTCTTTGCTAATTCGTTCATACTTCCGCTGTAAGTTGTCAG
 P  I  L  E  K  R  L  S  K  Y  E  G  D  I  Q  Q  S

      6410       6420       6430       6440       6450
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ACTTTACAGCTTTCTGGTTAGTAAAAGATACTGCTATCCATTTGTTCAAA
  E  M  S  K  D  Q  S  F  S  M  T  I  G  K  Q  V

      6460       6470       6480       6490       6500
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F  E  Q  R  A  E  A  G  E  S  L  H  R  L  I  R  H

      6510       6520       6530       6540       6550
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TTAGTTAGACTGTCGTTTCTTAAAGCTTGGGATCGTTCAATAGCTCCTAA
 N  Q  S  D  S  K  E  F  R  T  L  A  S  Y  R  G  F

      6560       6570       6580       6590       6600
TGACATTAAAATGCTTAGTCTTGCAACAAATCAACCTCTTCCTGAAACCT
ACTGTAATTTTACGAATCAGAACGTTGTTTAGTTGGAGAAGGACTTTGGA
  D  I  K  M  L  S  L  A  T  N  Q  P  L  P  E  T

      6610       6620       6630       6640       6650
TCTCTGTTAAGATTGTAGGAGAAAACCAATATTCTGTCAGTTTAGATTTG
AGAGACAATTCTAACATCCTCTTTTGGTTATAAGACAGTCAAATCTAAAC
F  S  V  K  I  V  G  E  N  Q  Y  S  V  S  L  D  L

      6660       6670       6680       6690       6700
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ATAAGAGGAAACCCCTGTTAAGTTTCCGAAGTCGTATGCTATCTGGTGTA
 Y  S  P  L  G  T  I  Q  R  L  Q  H  T  I  D  H  I

      6710       6720       6730       6740       6750
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ATTTCTACTGGTTCACTTTTGAGTCTTGAATAACCTACTTAATTTCCTAT
  K  D  D  Q  V  K  T  Q  N  L  L  D  E  L  K  D

      6760       6770       6780       6790       6800
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K  W  T  T  A  K  V  E  I  E  K  N  F  P  K  E  E
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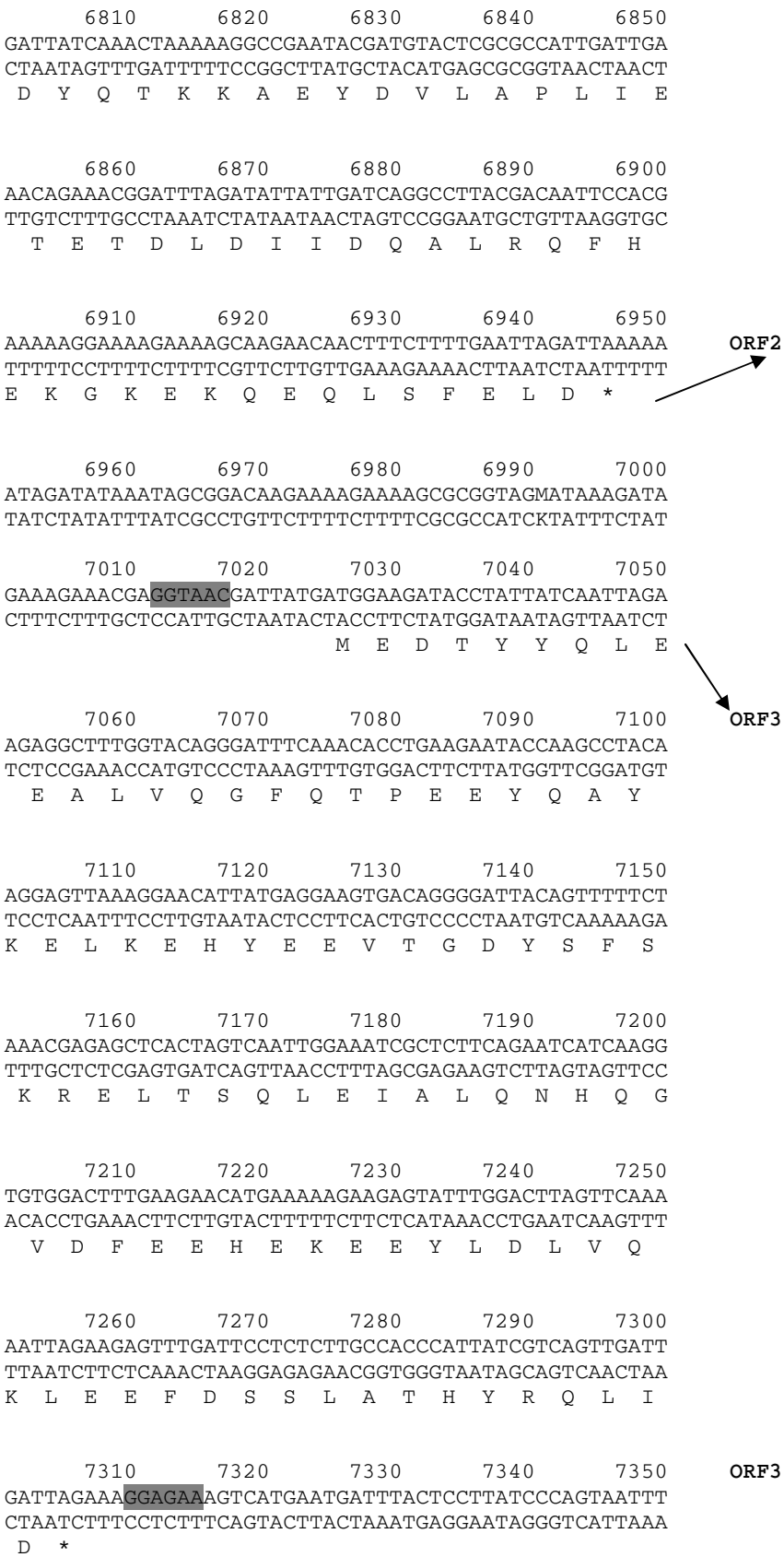
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D Y Q T K K A E Y D V L A P L I E
6860 6870 6880 6890 6900
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T E T D L D I I D Q A L R Q F H
6910 6920 6930 6940 6950
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E K G K E K Q E Q L S F E L D *
ORF2
6960 6970 6980 6990 7000
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7010 7020 7030 7040 7050
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CTTTCTTTGCTCCATTGCTAATACTACCTTCTATGGATAATAGTTAATCT
M E D T Y Y Q L E
7060 7070 7080 7090 7100
AGAGGCTTTGGTACAGGGATTTCAAACACCTGAAGAATACCAAGCCTACA
TCTCCGAAACCATGTCCCTAAAGTTTGTGGACTTCTTATGGTTCGGATGT
E A L V Q G F Q T P E E Y Q A Y
ORF3
7110 7120 7130 7140 7150
AGGAGTTAAAGGAACATTATGAGGAAGTGACAGGGGATTACAGTTTTTCT
TCCTCAATTTCCTTGTAATACTCCTTCACTGTCCCCTAATGTCAAAAAGA
K E L K E H Y E E V T G D Y S F S
7160 7170 7180 7190 7200
AAACGAGAGCTCACTAGTCAATTGGAAATCGCTCTTCAGAATCATCAAGG
TTTGCTCTCGAGTGATCAGTTAACCTTTAGCGAGAAGTCTTAGTAGTTCC
K R E L T S Q L E I A L Q N H Q G
7210 7220 7230 7240 7250
TGTGGACTTTGAAGAACATGAAAAAGAAGAGTATTTGGACTTAGTTCAAA
ACACCTGAAACTTCTTGTACTTTTTCTTCTCATAAACCTGAATCAAGTTT
V D F E E H E K E E Y L D L V Q
7260 7270 7280 7290 7300
AATTAGAAGAGTTTGATTCCTCTCTTGCCACCCATTATCGTCAGTTGATT
TTAATCTTCTCAAACTAAGGAGAGAACGGTGGGTAATAGCAGTCAACTAA
K L E E F D S S L A T H Y R Q L I
7310 7320 7330 7340 7350
GATTAGAAAGGAGAAAGTCATGAATGATTTACTCCTTATCCCAGTAATTT
CTAATCTTTCCTCTTTCAGTACTTACTAAATGAGGAATAGGGTCATTAAA
D *
ORF3

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                    M  N  D  L  L  L  I  P  V  I

      7360       7370       7380       7390       7400       ORF4
TTTTAGCAGTAGGAGGAATTATCATTCTTTTATGGAGACTCTTTCTTATT
AAAATCGTCATCCTCCTTAATAGTAAGAAAATACCTCTGAGAAAGAATAA
F  L  A  V  G  G  I  I  I  L  L  W  R  L  F  L  I

      7410       7420       7430       7440       7450
GCCAGTGGACTTTTCCTTATTGGTTTTGTCAGTTTTCTTATTTTCGTAGA
CGGTCACCTGAAAAGGAATAACCAAAACAGTCAAAAGAATAAAAGCATCT
 A  S  G  L  F  L  I  G  F  V  S  F  L  I  F  V  E

      7460       7470       7480       7490       7500
GGTCTATGGAATTTATCTGTTCTTTACTGAACCTAGTTTATATTTTGATG
CCAGATACCTTAAATAGACAAGAAATGACTTGGATCAAATATAAAACTAC
  V  Y  G  I  Y  L  F  F  T  E  P  S  L  Y  F  D

      7510       7520       7530       7540       7550
ATATCAGACAATATGGTTTAACTAGTTTTACGGCTGTGTACCTTTTCATC
TATAGTCTGTTATACCAAATTGATCAAAATGCCGACACATGGAAAAGTAG
D  I  R  Q  Y  G  L  T  S  F  T  A  V  Y  L  F  I

      7560       7570       7580       7590       7600
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TTAGACTACAACCAAGATCCTAAATCAACCGCGAAGTAATTGAGGTATTT
 N  L  M  L  V  L  G  F  S  W  R  F  I  N  S  I  N

      7610       7620       7630       7640       7650       ORF4
TAAGCATAAAATGTGAACTTTTTAGATAGTTAAAAGACTTTATTCAAAGG
ATTCGTATTTTACACTTGAAAAATCTATCAATTTTCTGAAATAAGTTTCC
  K  H  K  M  *

      7660       7670       7680       7690       7700
TAAGTTTGAATAAAGTCTTTTTGTTGATGTGCTTATAATCCCGTCTTAGT
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      7710       7720       7730       7740       7750
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TGAATCACACTCTTTGTTTTTCTCTACTTAATTTTAATTTCACATGTCTT

      7760       7770       7780       7790       7800
TGCTTAAGAGTTTCTATTTTTCTTATAATATATAGTAATTTCGGTACAAA
ACGAATTCTCAAAGATAAAAAGAATATTATATATCATTAAAGCCATGTTT

      7810       7820       7830       7840       7850
TTAACTTGTATATTTTATCTGGTTTAGTGTGATATATTCATTGTTAAATA
AATTGAACATATAAAATAGACCAAATCACACTATATAAGTAACAATTTAT
```

V583, Bucepa 03001966 from *Burkholderia cepacia* R1808, and cpp14 from *Campylobacter jejuni*. Most of these sequences listed are found associated with either plasmids or transposons.

Analysis of the deduced amino acid sequence of ORF2 showed the presence of two conserved domains, one for a helicase and one for a methylase (Fig. 3). Besides these two conserved domains, there was also a set of motifs which are usually found in the protein of the DEAD-box helicase family proteins. In Fig 4, the common sub-groups of helicases belonging to the DEAD-box helicase family are shown. By comparing the amino acid sequence between the residues 1278 and 1773, which contains the conserved motifs of DEAD-box helicase family (Fig. 4, ORF2 was found to belong to the DEAH* sub-group (NH_2--G-GKT---DEAH---A-------GR---COOH) which contain an ATP-biding motif (I), ATP-hydrolysis/nuclic acid-unwinding motif (II and III), and a nuclic acid binding motif (VI). The Ia, Ib, and V motifs found in protein of the DEAD-box helicase family were missing in ORF2 [93, 94]. DEAH* subgroup of proteins, such as the RAD3 of *Sacchromyces cerevisiae* and *Eco* KI HsdR of *E. coli* are DNA, rather than RNA, helicases [95]. The predicted structure of the ORF2 protein suggests that ORF2 could be a DNA helicase. However, experimental evidence to support this hypothesis is not available yet.

Introduction of the helicase operon into clinical pneumococci

To determine the function of the helicase operon during pneumococcal pathogenesis, we sought to introduce the operon from the non-encapsulated avirulent laboratory strain into a clinical encapsulated strain. However, filter mating, the traditional method for conjugation, was not able to create sufficient surface contact between the non-encapsulated strain and the encapsulated strain. Hence, the helicase operon was introduced into clinical strains by transformation as described below. This was accomplished by using an *E.coli* recombinant plasmid carrying the entire operon with surrounding transposon DNA.

Creation of Plasmid pSS141

Plasmid pSS141 was created previously as shown in Fig. 5 [5]. Briefly, an *E.coli* vector plasmid, pVA891 which does not autonomously replicate in streptococci, was inserted in the orientation shown in Fig. 5 into a pneumococcal lab strain with Tn*5252 Δ cat*. The chromosomal DNA was digested with *Kpn*I followed by a self-ligation. The self-ligated DNA was used to transform *E.coli* to obtain the plasmid, pSS141, carrying a 28 kb passenger DNA derived from coordinates 4.1 to 32.1 of Tn*5252*Δ *cat*.

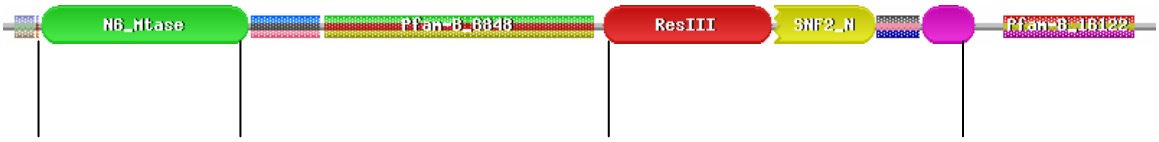


Fig 3. P-fam search on deduced amino acid sequence from ORF2. There are two conserved domains identified, the methylase domain, with e-value of $1.8*10^{-5}$ and DEAD-Box helicase domain with e-value of $6.2*10^{-5}$.

Organization and conserved motifs in DEAD-box family of helicases

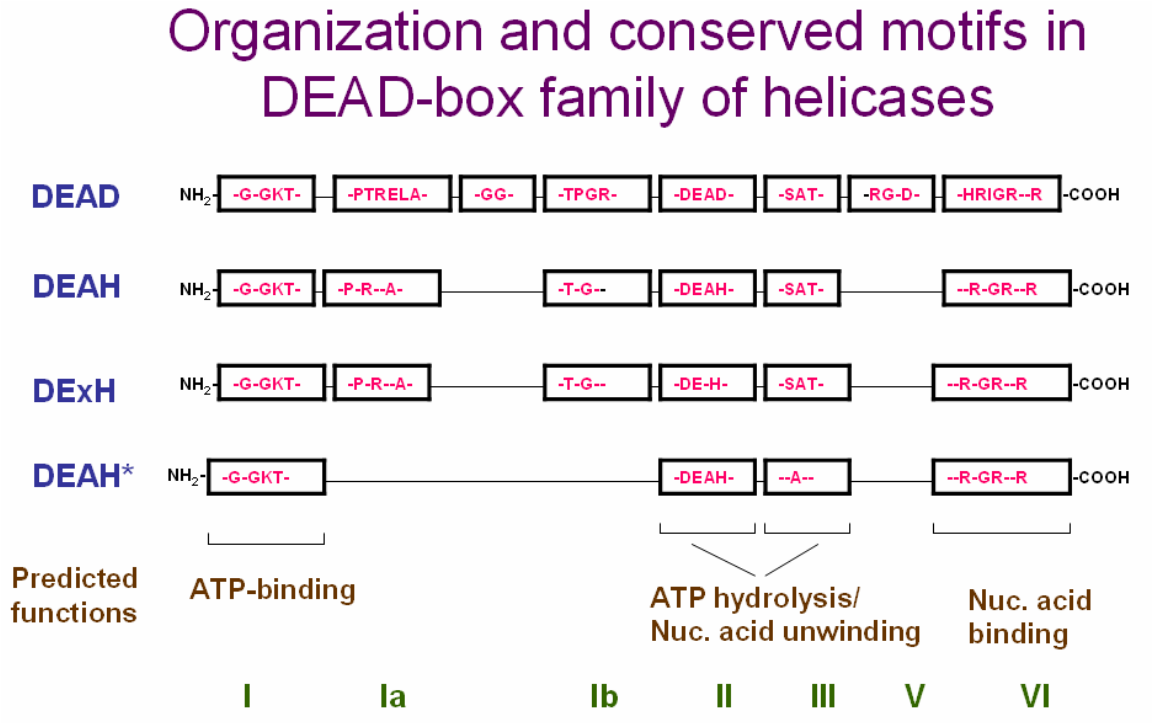


Fig 4. Conserved motifs in DEAD-box family of helicase [93]. There are 4 sub-groups in DEAD-box protein family. The DNA helicases belong to the DEAH* subgroup which lack the motifs, Ia, Ib and V.

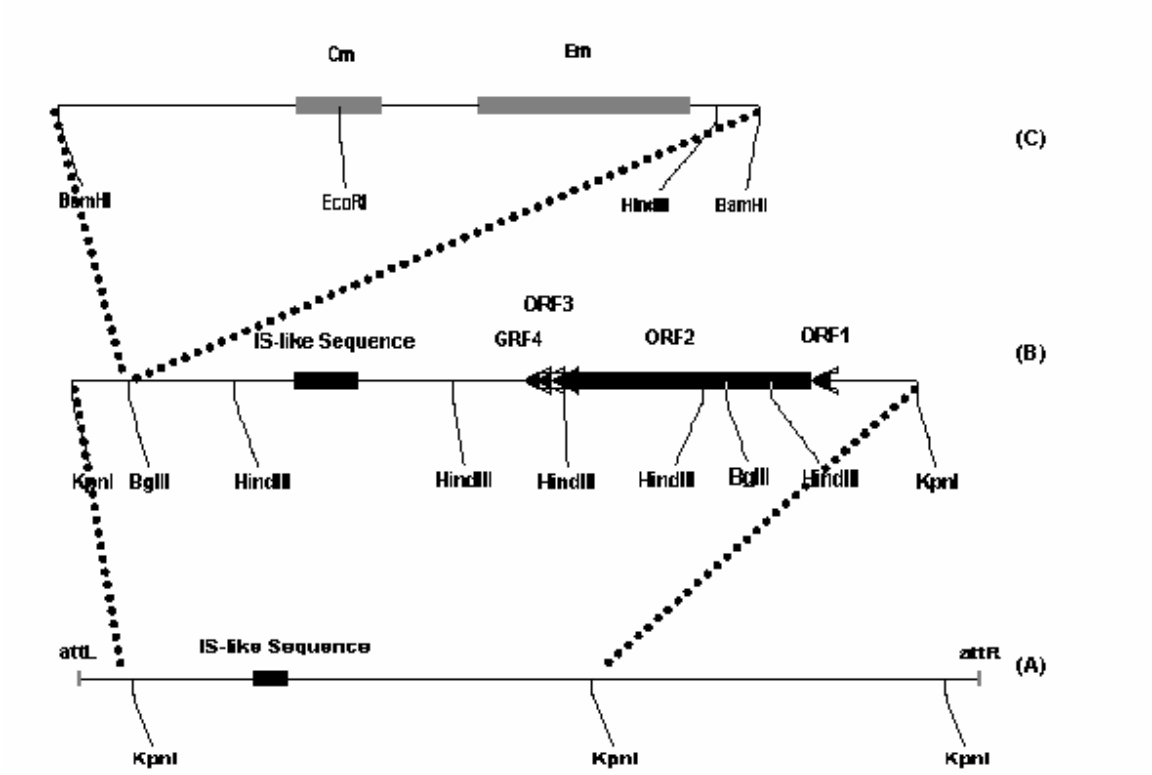


Fig. 5. Structure and partial restriction endonuclease map of the *E. coli* plasmid pSS141. (A) Tn*5252*Δ*cat*; (B) passenger DNA in pSS141; and (C) the *E. coli* vector plasmid pVA891. The location of the *IS*-like sequence and the ends of the element, *attL* and *attR*, are indicated. Relevant restriction sites are shown. Spontaneous curing of the 8 kb DNA carrying the chloramphenicol resistance determinant flanked by two directly repeated copies of the IS-like sequence, due to homologous recombination, led to the isolation of SP1403. The cryptic conjugative transposon, Tn*5252*Δ*cat*, in SP1403 carries a single copy of the IS- like sequence. *Bam*HI-digested pVA891 was inserted at the *Bgl*II site within the Tn*5252*Δ*cat* as shown in panel B. The chromosomal DNA from an Em^r transformant was digested with *Kpn*I, self-ligated, and used to transform *E. coli*. The resulting recombinant plasmid was designanted pSS141. The shaded arrows in panel B show the location and orientation of the four open reading frames in the helicase operon.

Creation of clinical pneumococcal strains for virulence assay

For convenience in the virulence assays, a genetic marker, *str,* was introduced into clinical wild type D39 (serotype II) and WU2 (serotype III) by transforming D39 and WU2 with DP1617 DNA and selecting for streptomycin resistance. D39 with *str* was named SP3001 and WU2 with the *str* was named SP3101. Similarly, SP3003 and SP3100 were obtained by introducing the *nov* marker into D39 and WU2.

Creation of clinical pneumococcal strains with the helicase operon

Introduction of the helicase operon into clinical strains was achieved by using pSS141 as donor DNA to transform SP3001 and SP3101. Plasmid vector, pVA891 [86], is not able to autonomously replicate in streptococci. This plasmid carries chloremphenicol and erythromycin resistance, but only erythromycin resistance is expressed in streptococci. In pSS141, there is an *IS*-like sequence, derived from Tn*5252*. We presumed that the chromosome of most pneumococci carried this element. When pSS141 was used as donor DNA to transform recipient clinical strains, as is shown in Fig. 6, the only way that recipient cell could gain resistance to erythromycin was through insertion of the entire pSS141 into the chromosome through insertion-duplication induced by the homology between the *IS*-like sequence in pSS141 and in the chromosomal DNA of clinical strains. As expected, pSS141 was successfully inserted into chromosomes of SP3001 and SP3101 to create SP3004 and SP3102, respectively. To create an insertion-mutation within the helicase operon, the plasmid, pZH12 was used. PZH12 was created

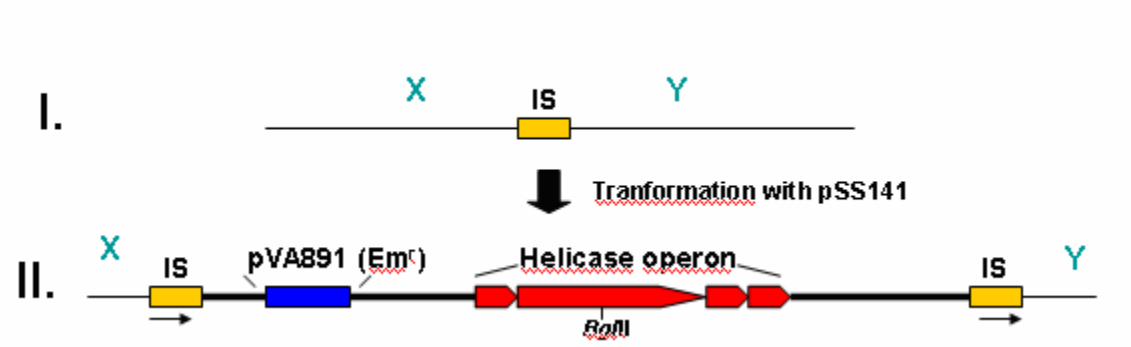
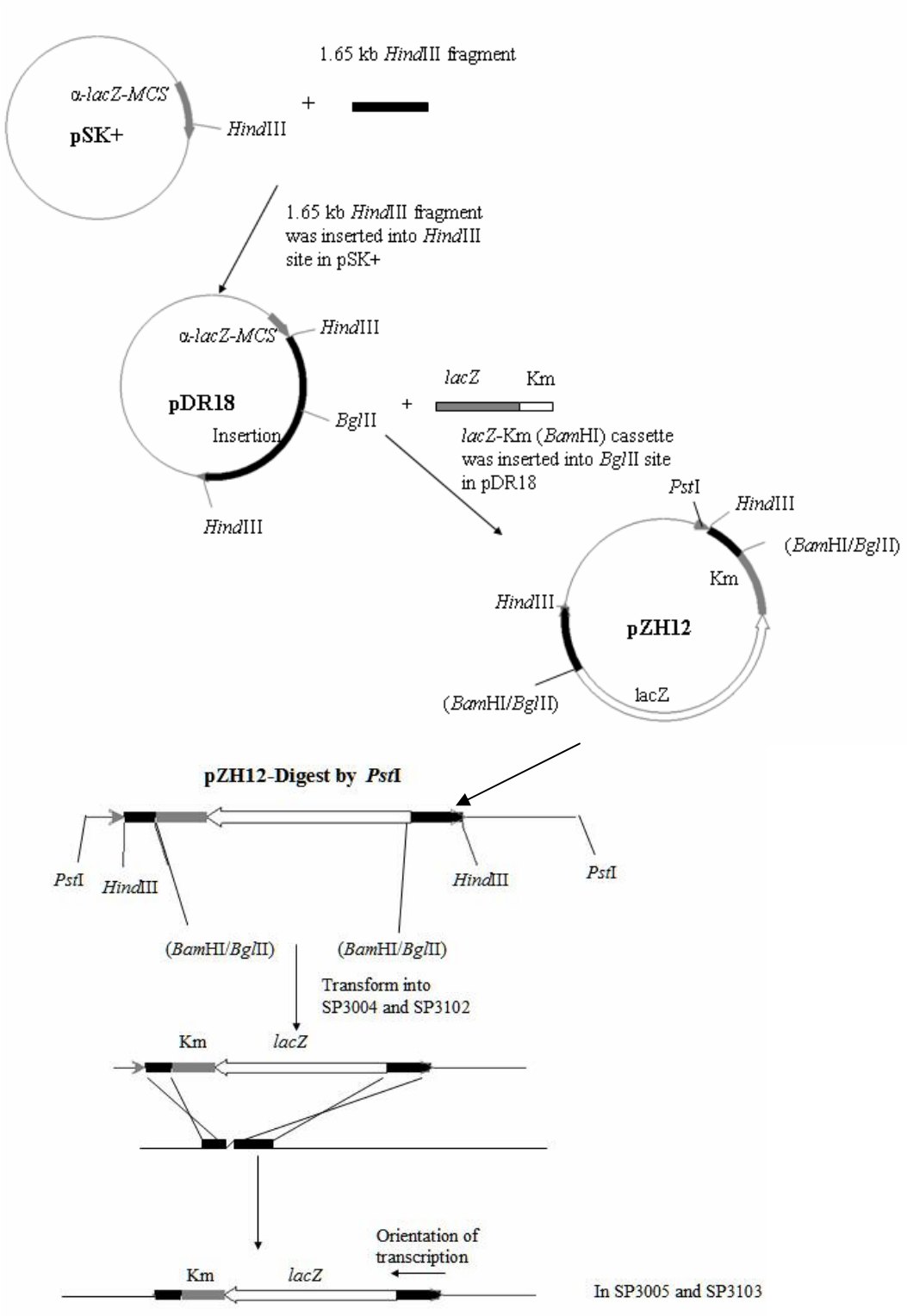


Fig. 6. Insertion of pSS141 into chromosomal DNA of clinical strains. I. As hypothesized and proved, the *IS*-likes sequence residing in pSS141 also resided in chromosome DNA of clinical strain. II. When intact pSS141 was used as donor DNA to transform clinical strain, the homology between *IS*-like sequence in pSS141 and *IS*-like sequence in chromosomal DNA of clinical strain would induce insertion duplication, in a process of which, entire pSS141 was inserted into chromosomal DNA of clinical strain, and *IS* like sequence was duplicated.

Fig. 7. Strategy for the creation of an insertion mutation in the helicase operon in SP3004 and SP3102



as shown in Fig. 7. Plasmid pDR18 consists of a 1.65 kb *Hin*dIII fragment of DNA from ORF2 cloned into plasmid pSK+. To create plasmid pZH12, a cassette containing the Km resistance gene, was obtained by *BamH*I digestion of pZH2 and was inserted into the unique *Bgl*II site in pDR18. Plasmid pZH12 was linearized with *Pst*I before it was used to transform SP3004 and SP3102. As is shown in Fig. 7, the homology between the sequence flanking the insertion cassette and the sequence in the host chromosome facilitated the insertion of cassette with Km resistance gene into the helicase operon in the bacterial chromosome, thus creating an insertion-mutation in the helicase operon. The new created strains from SP3004 and SP3102 were designated SP3005 and SP3103, respectively.

Southern hybridization to determine the integrity of inserted DNA in the newly created strains

Chromosomal DNA from SP3001, SP3004, SP3005, SP3101, SP3102 and SP3103 were isolated and analyzed in southern hybridization by using pSS141 as a probe to determine if the constructs in the chromosome of these strains were the same as expected (Fig. 8). Chromosomal DNAs from SP3001 and SP3101 showed hybridization to pSS141, indicating that the *IS* like sequence was present in the chromosome DNAs of SP3001 and SP3101 also. Since the genomic sequences of these two clinical strains were not available, it was impossible to predict the exact size of each band. However, the bands representing the helicase operon were not present in either SP3001 or SP3101, which indicated that the helicase operon was not present in these two strains. Insertion of

pSS141 into the recipient genome at the site of the *IS*-like element by insertion-duplication was expected to yield *Eco*RI fragments of 9.1, 4.6, 4.3, 2.6, 2.1, 1.9, 0.9, and 0.6 kb, and *Hin*dIII fragments of 6.9, 4.6, 3.3, 2.7, 1.7, 1.0, 0.3, and 0.2 kb. Similarly, insertion of the 5.3 kb *lacZ*-Kam^r cassette within the 1.65 kb *Hin*dIII in ORF2 was expected to additionally yield 9.2 and 5.2 kb *Eco*RI fragments and 5.2 and 1.8 kb *Hin*dIII fragments in SP3005 and SP3103. The band patterns observed confirmed that SP3004, SP3005, SP3102 and SP3103 each had the expected chromosomal structures.

Virulence studies

Since the colony morphology of clinical pneumococcus has been correlated with different capabilities to colonize various niches inside host[43], all strains to be used for infection were examined for colony morphology type. All the strains were found to be of the "transparent type" by the criteria described earlier.

To study the effect of the presence of the helicase operon in pneumococcal virulence, dilutions of cultures of each of the three strains in the D39 background were injected intraperitoneally into young female BALB/c mice. The mice were monitored at 12 hr intervals for four days to determine the mortality rate. As shown in Fig. 9, SP3004, the strain bearing pSS141 in the chromosome, resulted in a significantly fewer deaths than SP3001 (the parental strain) and SP3005 (the one with mutated helicase operon). Strains SP3001 and SP3005 resulted in similar mortality rates. As shown in Fig 9, the three strains in WU2 background also displayed similar results.

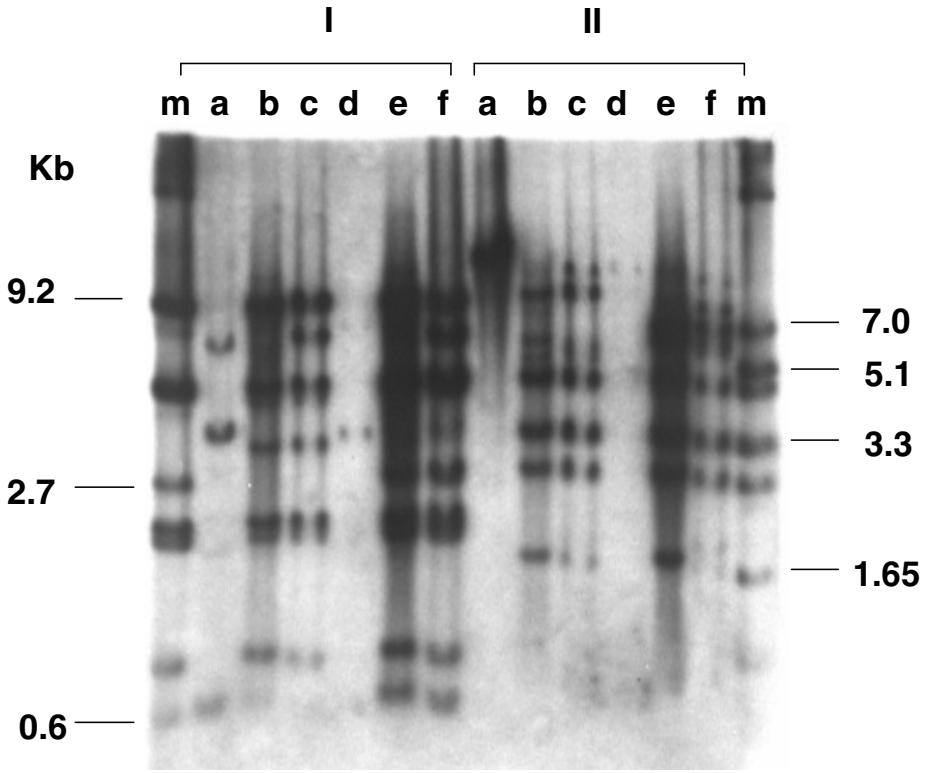


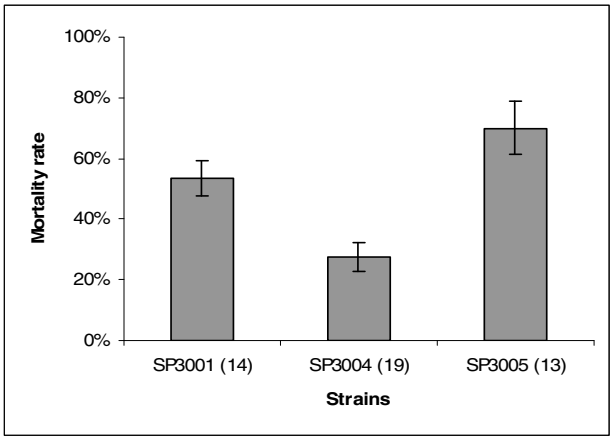
Fig 8. Physical analysis of chromosomal DNA of *S. pneumoniae* clinical strains showing the insertion of pSS141. Autoradiogram showing DNA-DNA hybridization of ^{32}P-labeled pSS141 to DNA from (a) SP3001, (b) SP3004, (c) SP3005, (d) SP3101, (e) SP3102, and (f) SP3103. *Eco*RI digests are shown in I and *Hin*dIII digests are shown in II. Lanes m, marker DNA containing linearized plasmids of known molecular weights that are expected to react with the probe DNA.

For the pneumonia model, isoflurane-anesthetized mice were intranasally infected with about 10^6 cfu (in 50 μl) of pneumococcal cells and monitored for four days (Fig. 10). When introduced into the nasopharyngeal region, SP3004 cell was able to cause significantly more death than SP3001 or SP3005. Similarly, SP3102 cells, caused more death than SP3101 or SP3103 when introduced by the nasal route (Fig 10). Even though all the infected animals showed morphological signs of disease such as staring coat, the mice nasally infected with strains carrying the intact operon succumbed more often than those infected with the other two strains. Thus, the presence of the helicase operon seemed to have an effect on the virulence of pneumococci depending on the route of infection. These results suggest that the helicase operon is activated *in vivo* in both routes of inoculation.

Competition Assays

To confirm the results obtained in the virulence assays, *in vivo* competition assays using a mixture cells with and without a functional helicase operon were performed. For intranasal challenge, a mixture of equal amounts of SP3003 (*nov*) and SP3004 (*str*) cells were used for infection. As a control, to estimate the effect of the antibiotic resistance markers used on bacterial fitness *in vivo*, mice were also separately infected with mixed cultures containing approximately equal amounts of the parental strains, SP3001 (*str*) and SP3003 (*nov*) [96]. During intranasal challenge, as is shown in Fig. 11, ultimately at 65 hours postinfection, SP3001 (a str^r derivative of D39) demonstrated as 4 times more efficiently than SP3003 (a nov^r derivative of D39) in surviving in nasopharyngeal region,

A.



B.

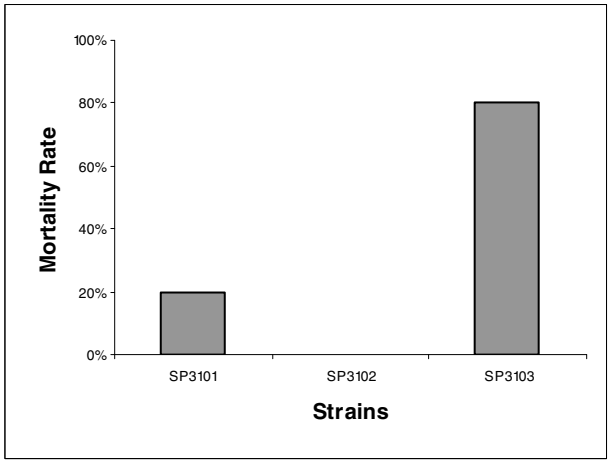
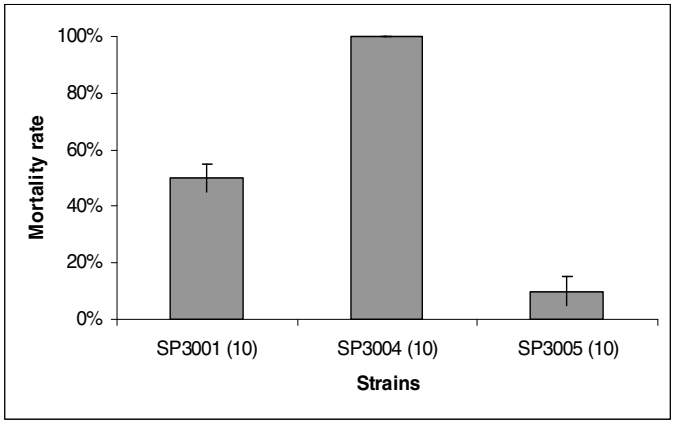


Fig. 9. Effect of the helicase operon on virulence of the indicated bacteria infected through the intraperitoneal route (A) D39 derivates (B) WU2 derivatives. Mortality was assessed periodically up to 72 hours post infection. The data presented in A is an average of three independent experiments each involving a minimum of five mice per group.

A.



B.

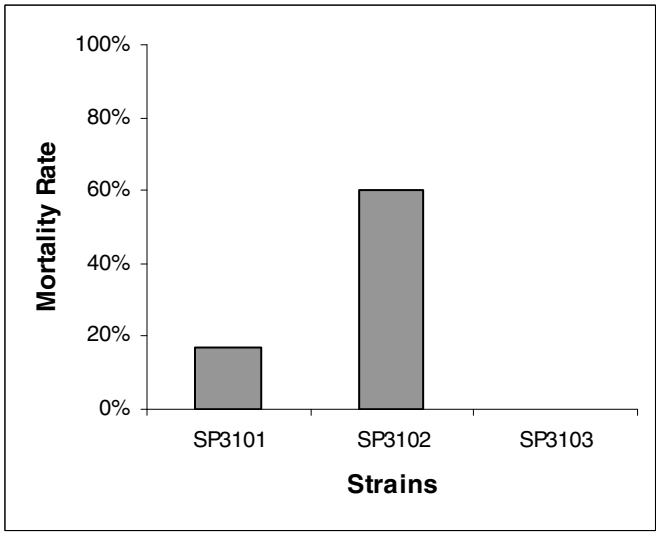


Fig 10. Effect of the helicase operon on virulence of the indicated bacteria infected through the intranasal route (A) D39 derivates (B) WU2 derivatives. Mortality was assessed periodically up to 72 hours post infection. The data presented in A is an average of three independent experiments each involving a minimum of five mice per group.

while SP3004 demonstrated as 20 times more efficiently than SP3003 in survival in the same region. Similarly, as is shown in Fig. 12, the same trend was identified with WU2 derivatives, SP3102 and SP3100.

During intraperitoneal challenge, as shown in Table V, mixed infections of SP3001 and SP3003 resulted in an 18-fold increase of SP3003 cells in comparison to SP3001 cells at 24 hours post-infection. Mixed infection of SP3003 and SP3004 resulted in 75-fold increase of SP3003 cells in comparison to SP3004 at 24 hours post-infection. This competitive edge of SP3003 was decreased when co-infected with SP3005 carrying an insertion mutation within ORF2 in the helicase operon. Similar results were obtained in competition experiments with genetically different strains, SP3102 and SP3100, as is shown in Fig. 13 which indicated a definitive role for the helicase operon in pneumococcal intraperitoneal infection. These results were in support of the data obtained from virulence assays in confirming that the presence of helicase operon differentially modulated the virulence of these strains depending on the route of infection.

On the other hand, the competition edge of a given strain, observed in intraperitoneal and intranasal infection, was not observed *in vitro* grown mixed cultures.

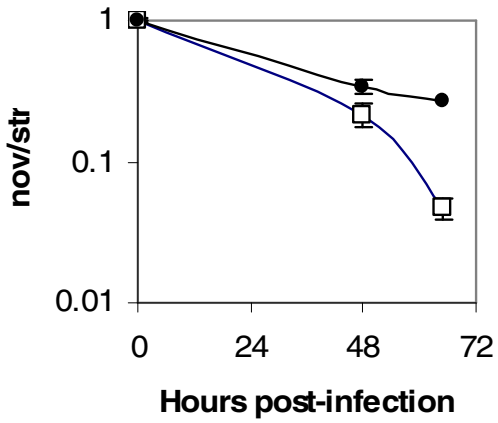


Fig. 11. Competitive indices for SP3003 (wt, *nov*) mixed either with SP3001 (wt, *str*, filled circles) or SP3004 (Ω helicase operon *str*, open squares) in nasopharyngeal colonization assays. The data shown for the pair of strains are derived from competitive infections of at least two mice. Error bars indicate standard deviations.

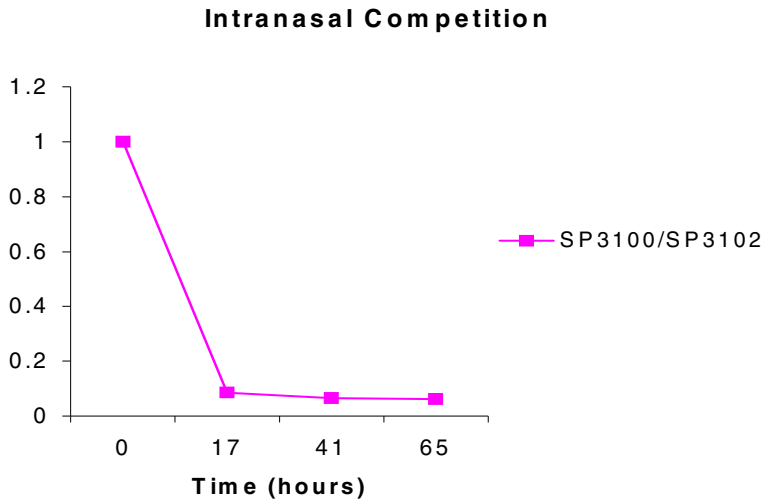


Fig. 12. Competitive indices for SP3100 (wt, *nov*) mixed with SP3102 (Ω helicase operon *str*) in nasopharyngeal colonization assays.

Study on induction of helicase operon

Insertion of reporter gene *lacZ* into ORF2 in helicase operon

For the purpose of inserting the *lacZ* reporter gene into the helicase operon, a fragment of DNA containing the whole ORF1 and part of ORF2 was amplified by PCR using primer G54-1 and G54-ORF1-F. Then the PCR fragment was then inserted into the *Sma*lI site in pEVP3 to create the *E. coli* plasmid, pZH38. The DNA sequence of the insert in the recombinant plasmid was obtained using the primers flanking the *Sma*lI site, pEVP3-F and pEVP3-R. The DNA sequence information confirmed that the transcriptional orientation of the inserted DNA was correct. The intact plasmid pZH38 was then used to transform pneumococcal strain SP1403 to create a chloramphenicol resistance strain, SP1472. SP1472 is the product of a transcriptional fusion of *lacZ* into ORF2 in the helicase operon through insertion-duplication. In SP1472, *lacZ* is expected to be transcribed and translated when transcription of operon becomes active.

Identification of conditions required for the induction of helicase operon

In order to determine the conditions required for the induction of the helicase operon, SP1472 was plated out in a chemical defined medium with X-gal. As shown in Table VI, colonies growing on agar plates supplemented with 50 μM Fe^{2+} turned blue after 72 hours of growth. Other divalent cations such as 50 μM Mn^{2+}, 50 μM Zn^{2+}, 50 μM MgCl, and 50 μM $CaCl_2$ or 0.1 M NaCl did not result in the activation of the operon and the colonies remained white for up to 2 weeks. To further study the effect of Fe^{2+}

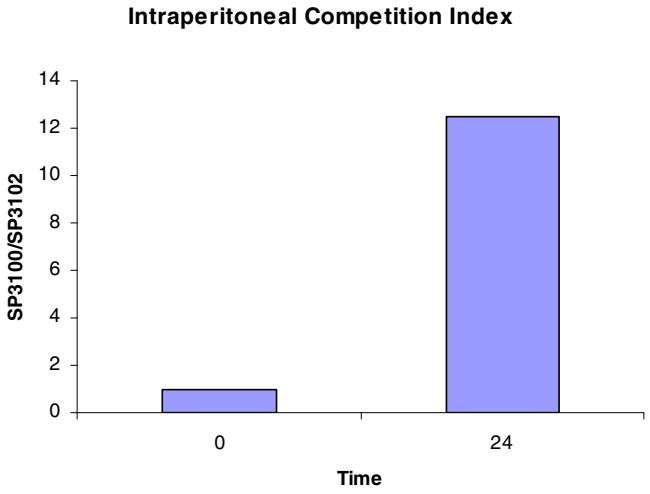


Fig. 13. Competitive indices for SP3100 (wt, *nov*) mixed either SP3102 *in vivo* and inoculated by intraperitoneal inoculation. Samples were collected at 0 and 24 hours post-infection.

TABLE V. Competitive indices for SP3003 (wt, *nov*) mixed with SP3001, SP3004 or SP3005 in vivo following intraperitoneal inoculation. Samples were collected in 24 hours post-infection.

Strains tested	Growth environment	*nov/str*[a]
SP3003 + SP3001	*In vitro*	0.67 ± 0.25
	In vivo	18.52 ± 2.95
SP3003 + SP3004	*In vitro*	1.35 ± 0.13
	In vivo	74.41 ± 15
SP3003 + SP3005	*In vitro*	0.33 ± 0.1
	In vivo	21 ± 2.82

[a]Data are the means ± standard deviations from two intraperitoneally infected mice or two independent mixed cultures grown *in vitro*.

concentration on the induction of the helicase operon, SP1472 was plated out in a chemical defined agar with X-gal and different concentrations of Fe^{2+}. It was found that at least 5 μM of Fe^{2+} can weakly induce the expression of the helicase operon. However, when SP1472 was grown in chemicaly defined broth, 5 μM Fe^{2+} led to no significant increase in β-gal Miller units. These results indicated that Fe^{2+} was only one of the factors required for induction of the helicase operon.

Effect of ORF1 on Induction of Helicase Operon

Since it was predicted that there was a calcium binding motif in ORF1 product, and transcription of the helicase operon was slightly induced by 5 μM Fe^{2+}, it was suspected that ORF1 may be involved in transcriptional regulation of the helicase operon. To determine the effect of ORF1 in transcriptional regulation of the helicase operon, the promoter region and promoter with ORF1 were each cloned separately into an *E.coli*-pneumococcus shuttle vector, pTCV-lac.

Creation of pZH48 and pZH49

The strategy used to create pZH48 and pZH49 is shown in Fig 14. Briefly, the promoter region of the helicase operon was amplified using primers VK109 and PmR-G54-orf1. The PCR product was cloned into the *Sma*lI site in pSK+. By sequencing, the orientation of the inserted the PCR product was determined. As shown in Fig 14, the clone picked contained a unique *Pst*I site in the vector portion upstream of the helicase

Table VI. Effect of divalent cation on induction of the helicase operon. Strain SP1472 was plated in chemically defined agar medium supplemented with X-gal in presence of the various cations listed below.

Condition tried	Color of Colonies [a]
50 μM Mn^{2+}	White
50 μM Zn^{2+}	White
50 μM $MgCl_2$	White
50 μM $CaCl_2$	White
0.1 M NaCl	White
50 μM Fe^{2+}	Blue

a. Colony color was observed after 72 hours.

operon promoter and this vector designated pZH46. Similarly, sequence containing the promoter region of the helicase operon and ORF1 was amplified using primers VK109 and G54-1 and the PCR product was inserted into the *Smal*I site in pSK+. After determination of the orientation of the insert by sequencing, a clone was picked and named pZH47. By inserting a spectinomycin resistance cassette, predigested by *Pst*I, into the unique *Pst*I site of pZH46 and pZH47, pZH48 and pZH49 were created.

Creation of pVJ603 and pVJ604 and Assay of SP1516 and SP1517

The *E.coR*I-*Bam*HI fragment from pZH48 and pZH49 was purified and inserted into pTCV-lac, which was digested with *E.coR*I and *Bam*HI, to create pVJ604 and pVJ605, respectively. Plasmids pVJ603 and pVJ604 were then used to transform pneumococcal β-gal$^-$ strain CP1250 to create SP1516 and SP1517, respectively. When SP1516 and SP1517 were plated in a chemically defined medium with 5 μM Fe^{2+} and X-gal, colonies from both turned blue after 72 hours. However, when they were grown in chemically defined medium with 50μmol Fe^{2+} supplement, no β-gal Miller unit was obtained from either of them. These results indicated that ORF1 in the helicase operon was unlikely to be involved in regulating of the helicase operon.

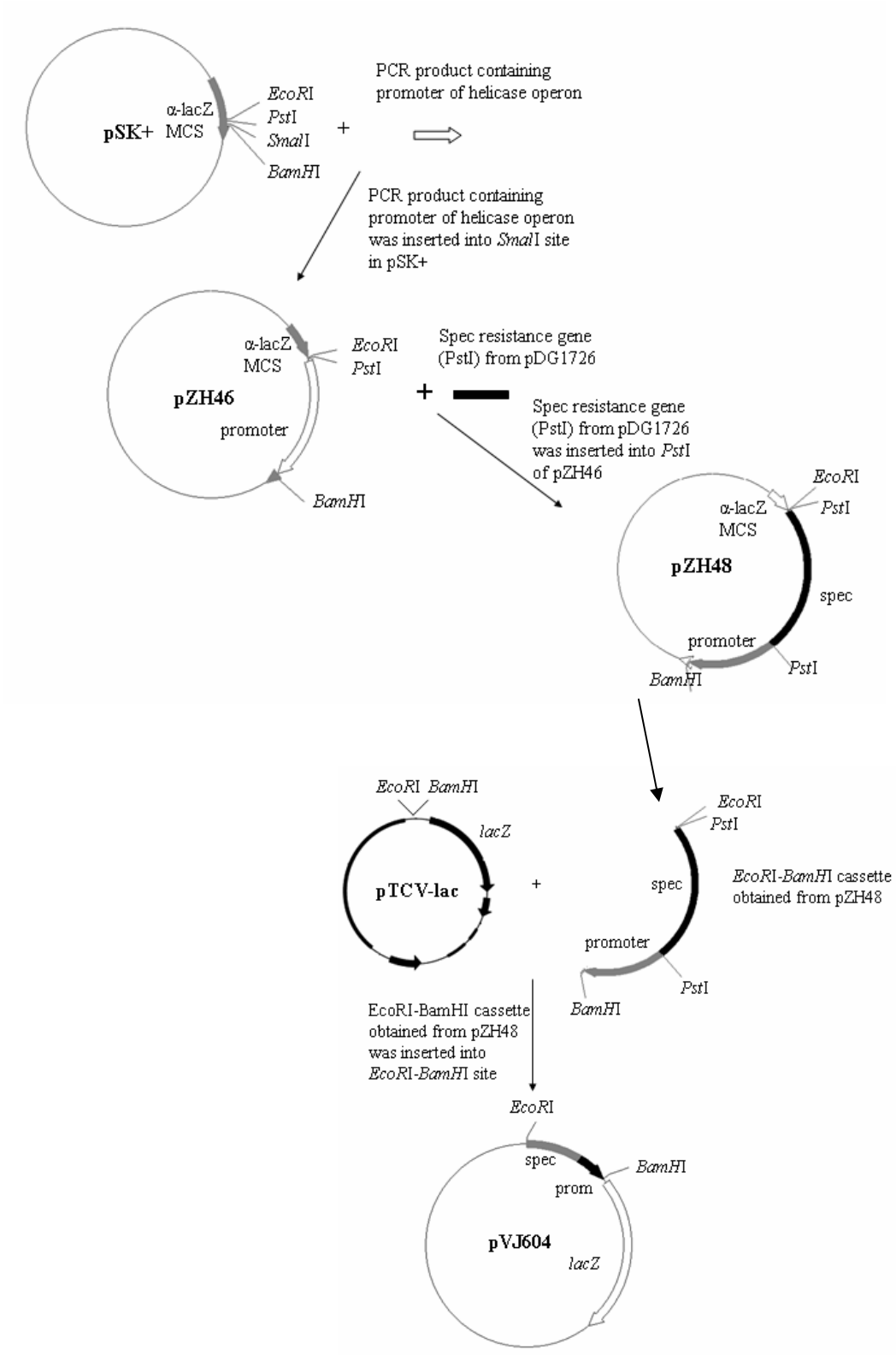


Fig. 14-a. Strategy for the creation of pVJ604

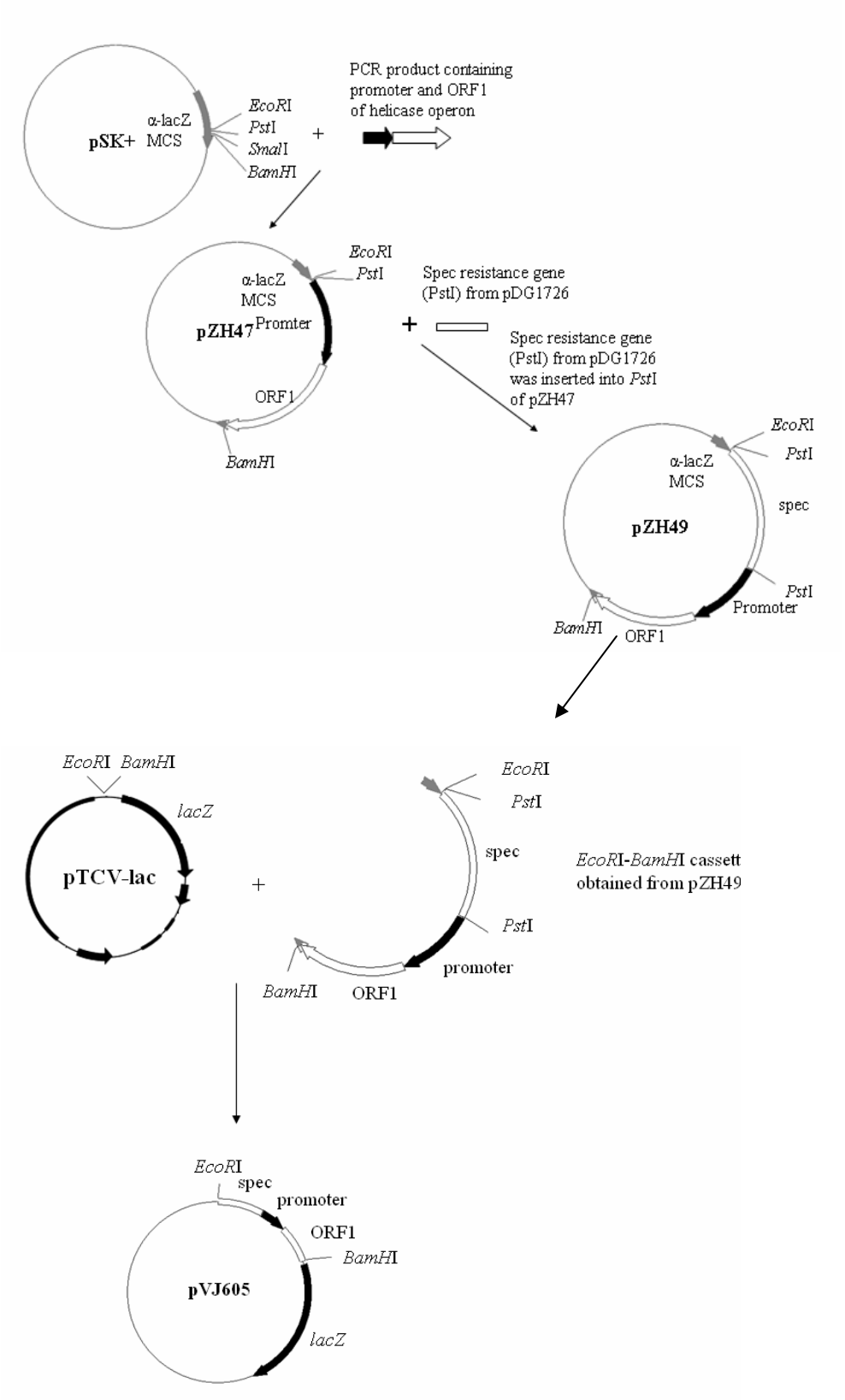


Fig. 14-b Strategy for the creation of pVJ605

Creation of a pneumococcus strain with the helicase operon controlled by the inducible promoter P_{malX}

Analysis of the deduced amino acid sequence from the helicase operon and predicted cytoplasmic localization of ORF1, ORF2 and ORF3 implied that the protein products of these three open reading frames could be involved in regulation of other virulence related genes. Limited understanding on the induction of this operon made it difficult to directly study the function of this operon. In order to solve this problem, it was useful to insert an inducible promoter in between the natural promoter and ORF1. It was reported that transcription driven by the maltose promoter, $P_{malx,}$ was regulated by maltose [97]. So in our study, we inserted the maltose promoter P_{malx} upstream of ORF1 in the helicase operon, so that the expression of this operon would be activated by maltose supplemented in medium.

Creation of SP1407

Dr Vijayakumar amplified the gene encoding the maltose repressor, *malR*, from chromosomal DNA using primers MalR-F and MalR-R. The PCR product was digested with *EcoR*I and inserted into the *E.coR*I site in pLS1. This plasmid was named pVJ572. Plasmid pVJ572 was then used as donor DNA to transform SP1403, a pneumococcal β-gal$^-$ strain. The new strain was named SP1407

Creation of pVJ595

To make it convenient in later transformations, a construct, in which *MalX* promoter was tagged with an antibiotic resistance gene, was created as shown in Fig. 15. The *MalX* promoter from chromosomal DNA was amplified using primers Pm-Mal3 and Pm-Mal4. The PCR product was inserted into the *Sma*lI site in pDL278-D. The new plasmid was named pVJ587. An erythromycin resistance cassette, obtained from pDG647 digested with *Hind*III, was then inserted into the *Hind*III site in pVJ588. The new plasmid was designated as pVJ595.

Insertion of P_{malX}-Em^r upstream of the helicase operon by crossover PCR

For crossover PCR, as shown in Fig 16, the P_{malX}-Em^r cassette was amplified from pVJ595 by using the primers, 647 Em-F and Pm-Mal3. A piece of DNA upstream of the insertion site was amplified from chromosomal DNA by using primers VK107 and VK108. Also, a piece of DNA downstream of insertion site was amplified from chromosomal DNA using the primers G54-1 and VK110. In VK110, there were 17 bases in the 5' end complementary to 3'-end of the malX promoter. Similarly, in VK107, there were 17 bases at the 5' end that were complementary to the 3'-end of the Em resistance gene. In the second step, the three PCR products obtained in the first step were mixed together and amplified by using the primers from the terminal ends, G54-1 and VK109, to obtain one fragment of 3.5 kb containing of all three input DNAs. Then the large fragment was purified and used as donor DNA to transform the pneumococcal strain

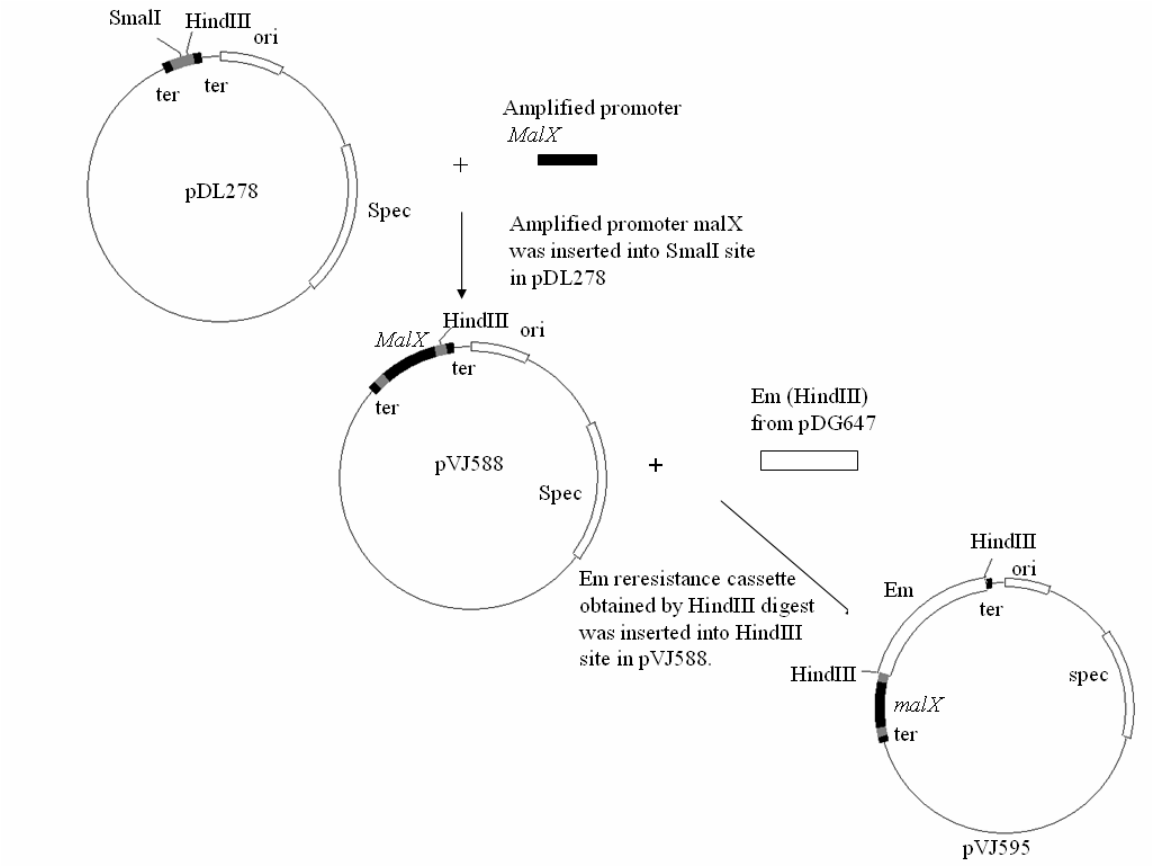


Fig. 15. Strategy for the creation of pVJ595. In pVJ595, the malX promoter was tagged with the antibiotic resistance gene, Em^r, which provided a selection marker for introduction of malX promoter into pneumococcus.

SP1407. The newly created strain was named SP1460. To create a pneumococcal strain with the inducible promoter malX in a strain without pVJ572, chromosomal DNA from SP1460 was prepared and used as donor DNA to transform SP1403 to create the strain SP1519.

Southern Hybridization to determine the insertion of malX Promoter

Chromosomal DNA from SP1460 and SP1000 were prepared and analyzed by Southern hybridization was performed using the probe pZH22, in which a 6 kb *Sst*I fragment from the center region of Tn*5252* was cloned into pAT29. Prior to the insertion of P_{malX}, the helicase operon in Tn*5252* is expected to have a 6.0 kb *Sst*I fragment; 1.65 kb, 3.33 kb and 3.59 kB *Hind*III fragment; 7.32 kb band *Bam*HI fragment; 17.5 kb band *EcoR*I. With the insertion of P_{malX}-*Em* cassette, the helicase operon in Tn*5252* is expected to have 4.67 kb and 3.13 kb *Sst*I fragments; 1.65 kb, 1.47 kb, 3.33 kb and 3.92 kb of *Hind*III fragments; 3.6 kb, and 5.5 kb bands of *Bam*HI fragments; and 6.25 kb and 12.52 kb of *EcoR*I fragments. As shown in Fig. 17, the hybridization pattern of SP1460 DNA indicated that the P_{malX}-Em cassette was inserted giving rise to the expected bands. The patterns seen with SP1000 (negative control) and another clone, SP1437, indicated that there was no *malX*-Em cassette inserted upstream of the helicase operon. Hence SP1460 was used in further experiments.

Induction Assay on SP1474

Though results obtained from Southern hybridization proved that SP1460 was the expected construct, to further confirm that the helicase operon in SP1460 will be induced as expected, we introduced the transcriptional fusion, *lacZ* into ORF2 in the helicase operon. Plasmid DNA pZH38 was used as donor DNA to transform SP1460, the strain with helicase operon controlled by P_{malX} promoter. The new strain was named SP1474. When SP1474 was plated out in CATPM (the same concentration of maltose substituted for glucose as in CATPG) and X-gal, colonies gave a dark blue color on the second day. Similar results were obtained when the reporter gene was inserted into strain SP1519. Then SP1474 was grown in CATP with 1% (w/v) glucose, 1% (w/v) maltose, 2% maltose, 4% maltose, 6% maltose and 8% maltose, respectively. Miller β-galactosidase acitvity was measured in each sample. As shown in Table VII, SP1474 showed some leaky expression when it was grown in glucose, in comparison to the negative control. When it was grown in the same concentration of maltose, there was about 2.5 times induction. However with the increase of maltose concentration, there was no significant increase of β-gal activity.

Global Search for genes influenced by the helicase operon

Results from the induction assay of SP1474 confirmed that in strain SP1460, expression of the helicase operon was induced as expected by maltose. To globally search for genes potentially influenced by the helicase operon, the reporter gene *lacZ* was used

to randomly mutagenize the chromosome of SP1460. The strategy employed is shown Fig. 18. In brief, chromosomal DNA from CP1250 was prepared and digested with *Mbo*I. The digested DNA was ligated into pEVP3 digested with *BamH*I. The ligation mixture was used to transform *E.coli* competent cells and Cm^r transformants were obtained. All the colonies obtained were pooled together and used to prepare plasmids. The plasmid pool was then used to transform the pneumococcal strain, SP1460. The transformants were initially plated out on CATPG agar with X-gal. Then they were replicate-plated on CATPG with X-gal and CATPM with X-gal to screen the clones which gave blue color in CATPG but white color in CATPM or vice versa. Altogether, there were more than 5,000 clones screened, however, none of them appeared to give differential color patterns.

Effect of the induction of the helicase operon on pneumococcal survival during oxidative stress

The results from induction of the helicase operon show that Fe^{2+} is one of the factors required for induction. It has been reported that during Fenton's reaction, Fe^{2+} is able to react with hydrogen peroxide, a by-product generated during pneumococcal aerobic growth, to generate hydroxyl radicals which are harmful to pneumococcal cells [98]. For this reason, it was suspected that induction of the helicase operon may have an effect on pneumococcal survival in the presence of hydrogen peroxide

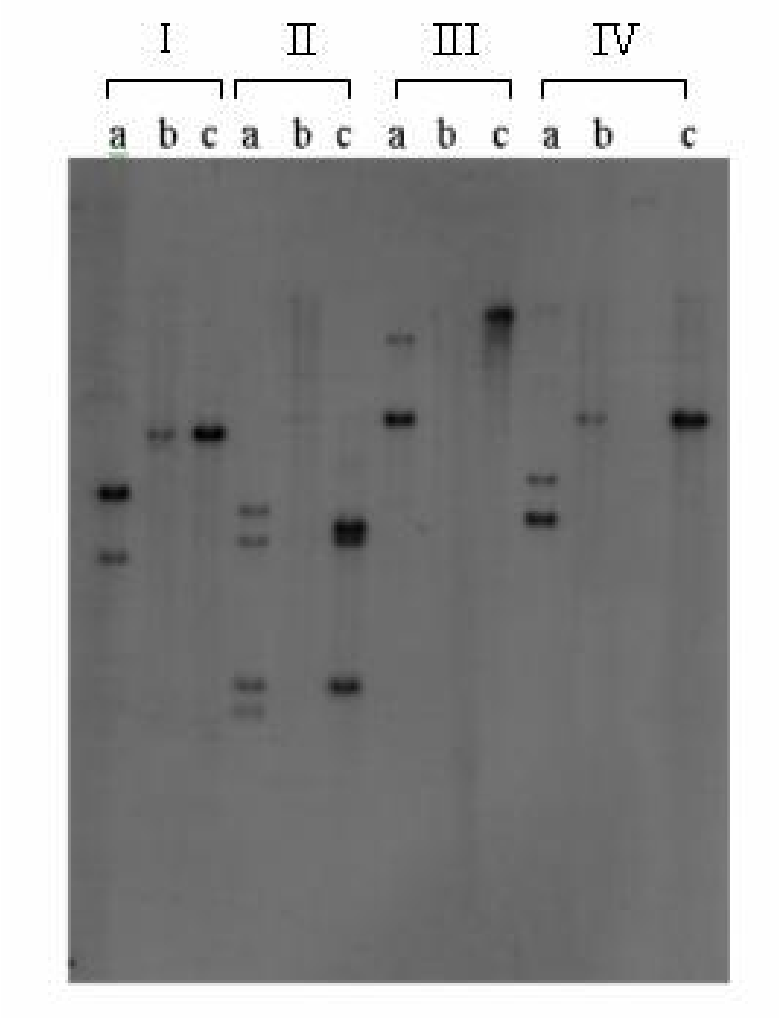


Fig. 17. Southern hybridization analysis of the strains with the *MalX* promoter inserted upstream of helicase operon. Autoradiogram showing DNA-DNA hybridization of ^{32}P-labeled pZH22 to DNA from (a) SP1460, (b) SP1437, and (c) SP1000 digested by *Sst*I (I), *Hind*III (II) *Eco*RI (III), and *Bam*HI (IV).

Table. VII Induction assay on the strain with the *malX* controlled helicase operon. Strain SP1474 was grown in CATP with specific carbohydrate listed below.

Strains/ Conditions	SP1474/1% glucose	SP1474/1% maltose	SP1474/2% maltose	SP1474/4% maltose	SP1474/6% maltose	SP1000/1% glucose	SP1000/1% lactose
β -gal Unit[a]	1.38±0.49	3.15±0. 24	2.89±0. 08	2.79±0. 36	2.55±0. 18	2.99±0. 61	18.79±2. 05

a. Data are the means ± standard deviations from three independent experiments.

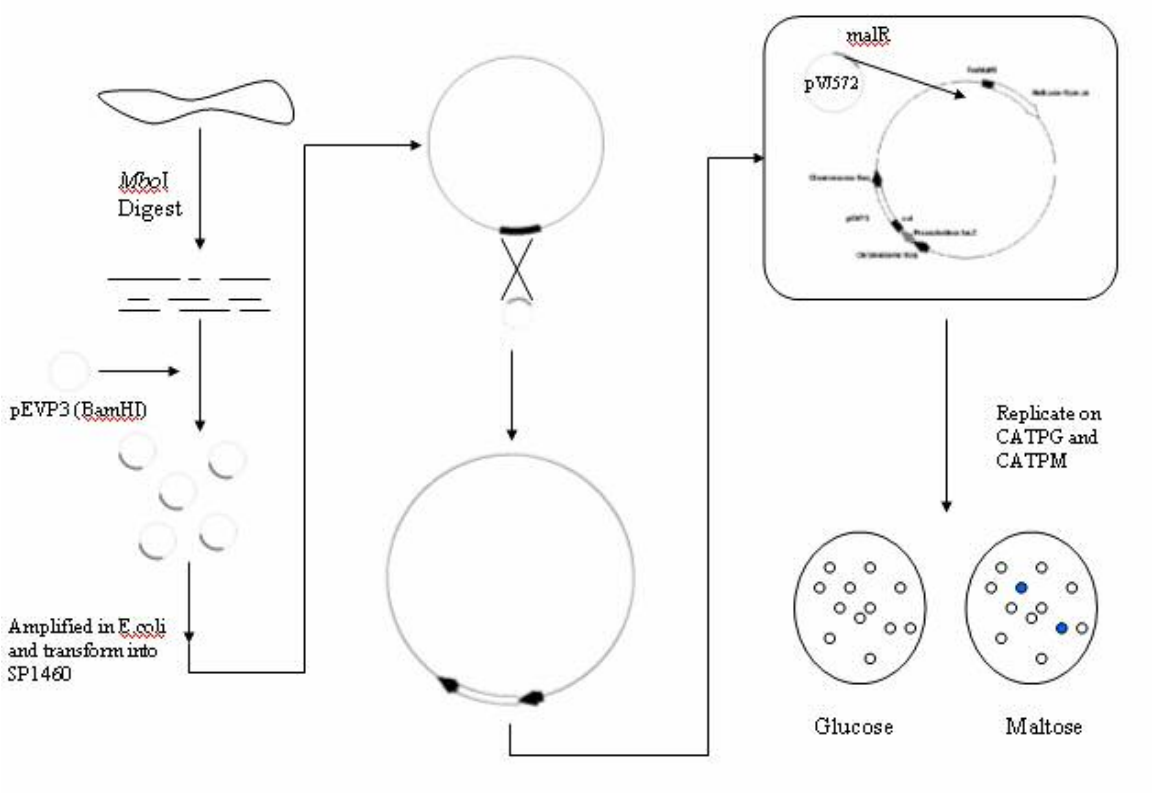


Fig. 18. Strategy to randomly insert the reporter gene *lacZ* into the chromosome of strain SP1460. Chromosomal DNA was cut into pieces with *Mbo*I before it was ligated with pEVP3 (*Bam*HI). The recombined plasmids were amplified before they were used to transform SP1460, in which helicase operon was under the control of P_{malX}. Each transformant would have reporter gene *lacZ* inserted into one locus in chromosome of SP1460. All the transformants were then replicate-plated on CATPG and CATPM respectively.

To determine the effect of the helicase operon on pneumococcal survival after exposure to hydrogen peroxide, strains SP1000, SP1519, Rx1 and SP1460 were grown in CATP with 22 mM maltose prior to hydrogen peroxide susceptibility assays. As shown in Table VIII, in static culture, comparing to Rx1 and SP1000, both SP1460 and SP1519 were 10-fold more sensitive to hydrogen peroxide. However, there were no significant difference between SP1460 and SP1519, or SP1000 and Rx1.

Also in Table 3-6, when the cultures were aerated for 20 minute before assay, each strain became at least 10-fold more sensitive than static culture.

Effect of various concentrations of hydrogen peroxide on the survival of SP1000 and SP1519

To confirm the results obtained from hydrogen peroxide sensitivity assay, SP1519 pre-grown in 22 mM maltose was treated with 2 mM, 4 mM, 6 mM, 8 mM and 10 mM hydrogen peroxide. As shown in Fig. 19, the lowest concentration to have a lethal effect on SP1000 was between 2 mM and 4 mM while for SP1519, it was between 0 to 2 mM. These results confirmed that an active helicase operon made pneumococcal cells more sensitive to hydrogen peroxide.

Effect of the active helicase operon on the survival of pneumococcus following treatment with other oxidative reagents

Results from hydrogen peroxide sensitivity assays show that induction of the helicase operon makes pneumococcal cell more sensitive to hydrogen peroxide. This result suggests two other possible oxidative inhibitors superoxide dismutase (*sodA*) [98] and pyruvate oxidase (*spxB*) [15]. If it is true that the helicase operon down-regulates expression of *sodA*, a strain with an active helicase operon should also be sensitive to other oxidase reagents. To determine the effect of the helicase operon on survival of pneumococcus in the presence of other oxidative agents, SP1519 were pre-grown in 22 mM maltose, and treated with methyl viologen or tert-butyl hydroperoxide respecitvely. As shown in Table IX, after treatment with either methyl viologen or tert-butyl hydroperoxide, there were no significant difference in survival rate between SP1000 and SP1519.

Effect of the helicase operon on transcription of pyruvate oxidase

Since the possibility that the helicase operon would affect the expression of sodA was ruled out, we wanted to determine if the helicase operon has an effect on the expression of *spxB*.

LacZ transcriptional fusion to pyruvate oxidase

To create transcriptional fusion of *lacZ* to the gene encoding pyruvate oxidase (*spxB*), a fragment of DNA completely internal to *spxB* was amplified from chromosomal DNA using primers VJ115 and VJ116. The PCR product was inserted into pEVP3 (*Sma*lI) to create *E. coli* plasmid pZH54. The recombined plasmid was sequenced by using the primers flanking the *Sma*lI site, pEVP3-F and pEVP3-R, to confirm that the transcriptional orientation of the inserted DNA was the same as *lacZ* in pEVP3. To create SP1525 and SP1528, the intact plasmid pZH54 was used as donor DNA to transform pneumococcal strains SP1403 and SP1519, and chlorenphenicol was used as selection.

Examination of insertion of *lacZ* in pyruvate oxidase

When SP1525 and SP1528 were sub-cultured in CATPG supplemented with BSA, about 8 hours of prolonged lag phase was observed. This prolonged lag phase disappeared when sodium acetate was supplemented in the medium. When SP1525 and SP1528 were spread on the surface of CATPG agar, their growth on the agar surface did not require the presence of pre-added catalase. All of these characteristies perfectly met the previously described characteristics of *spxB* mutant pneumococcus[14].

TABLE.VIII. Effect of the helicase operon on the survival of pneumococci in hydrogen peroxide.

Growth condition prior to H_2O_2 exposure*	Rx1	SP1000	SP1460	SP1519
Static	9.1 ± 6.07	14.7 ± 4.24	1.1 ± 0.78	1.88 ± 0.78
Aeration for 20 min.	0.06 ± 0.01	0.07 ± 0.05	0.012 ± 0.02	0.025 ± 0.01

* The cells were grown in 22 mM maltose to mid-exponential phase and divided into two aliquots. One continued to be grown under static conditions. The other was grown shaking at 200 rpm for 20 minutes at 37^{o}C following which both were exposed to 10 mM H_2O_2 for 30 minutes at 37^{o}C under static conditions prior to plating on agar plates to select for survivors. The data given is an average of survival rates ± standard deviation of three independent assays.

Table IX. Effect of the helicase operon on pneumococcal survival in oxidative stress

	methyl viologen	tert-butyl hydroperoxide
SP1000	30.35%±5. 16	42.90%±3. 39
SP1519	37.30%±0.28	47.20%±6.50

Cells were grown in 22 mM maltose to mid-exponential phase and divided into two aliquots. They were exposed to either 30 mM methyl viologen or 40mM tert-butyl hydroperoxide for 30 minutes at 37^{o}C under static conditions prior to plating on agar plates to select for survivors. The data given is an average of survival rate± standard deviation of three independent assays.

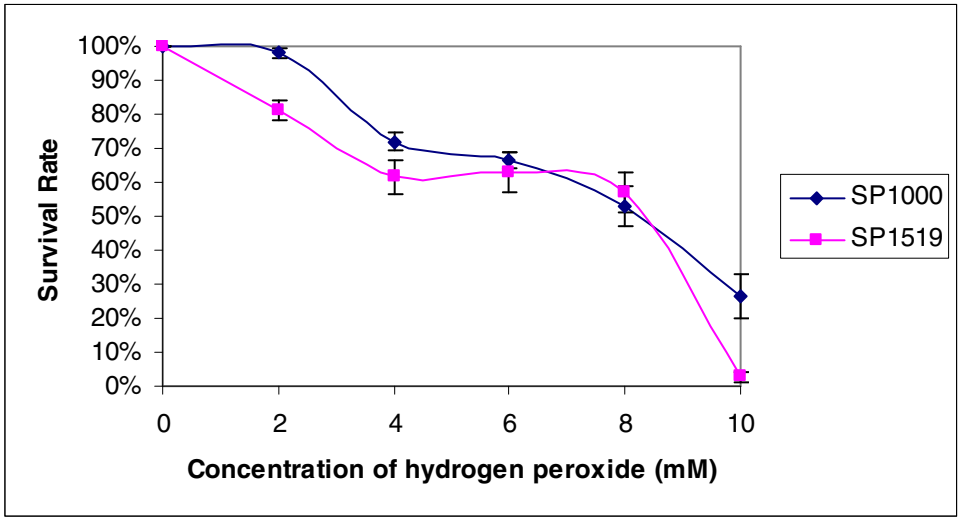


Fig. 19. Death curve of pneumococcus in a series of concentrations of hydrogen peroxide. Hydrogen peroxide susceptibility assays were carried out separately using strains SP1000 and SP1519 in the concentrations of hydrogen peroxide listed in figure. The error bar represent the standard deviation of results from two independent experiments.

Effect of the helicase operon on the transcription of pyruvate oxidase in static and aerated culture

To determine the effect of the helicase operon on transcription of *spxB*, SP1525 and SP1528 were grown in 22 mM maltose before β-galactosidase assay. As shown in Table XI, after aeration, there was about a 70% increase in the transcription level of *spxB* in both strains. By comparing SP1525 and SP1528, it was shown that the helicase operon led to about a 30% increase in transcription of *spxB* in static culture. A similar level of increase was also observed in culture after aeration.

Effect of the helicase operon on hydrogen peroxide production

It has been reported that up-regulation of spxB would lead to more production of hydrogen peroxide. Since we figured out that inducing the helicase operon leads to increased transcription of spxB [15], we wanted to determine if the production of hydrogen peroxide is increased accordingly.

Making a Standard Curve for Hydrogen Peroxide Production Assay

To make standard curve, a hydrogen peroxide stock solution was diluted in CAT to a series of concentrations. By using these samples in an assay, a standard curve was generated. As shown in Fig. 20, the formula for the curve was $y = 0.39x - 0.1327$ with R^2

= 0.9993, in which the Y-axis represents the absorbance at 560_{nm} and the X-axis for hydrogen peroxide concentration in mM.

Effect of the helicase operon on hydrogen peroxide production in static vs aerated cultures

Pneumococcal strain SP1519 was grown in CATP with 22 mM maltose before cells were processed and used in assays. As is shown Table X, it was observed that cells after aeration produced about 10-fold more hydrogen peroxide than static cultures. However by comparing SP1000 and SP1519, neither in static culture nor in aerated culture, was there a significant difference observed in hydrogen peroxide production between SP1519 and SP1000.

Effect of the helicase operon on natural autolysis of pneumococcus

Besides the effect on expression of *spxB*, we wondered if the helicase operon had any effect on the other pneumococcal phenotypes. To determine the effect of the helicase operon on natural autolysis, SP1519 was grown in CATPM. Time point 0 was set when OD_{550nm} of the culture reached 0.4. At this time, the culture was diluted and plated out for viable count. Then at 3 hours, 5 hours and 8 hours, samples were obtained and plated for viable counts. As shown in Fig 21, by comparing viable counts, there was no significant

difference observed at 3 and 5 hours between SP1000 and SP1519. However, at 8 hours SP1000 showed more than 4 times of surviving cells as compared to SP1519.

Effect of carbohydrate on pneumococcal survival in hydrogen peroxide

It has been reported that pneumococcus is able to metabolize more than 20 types of carbohydrates. In our study of the on helicase operon, we used maltose as an inducer to activate the helicase operon. During the hydrogen peroxide sensitivity testing, we happened to figure out that by comparing with glucose and maltose, lactose is able to give pneumococcus an exceptional higher resistance to hydrogen peroxide.

To determine the effect of carbohydrate on survival of pneumococcus in hydrogen peroxide, pneumococcal strain DP1004 was grown in CATP with 1% (w/v) various carbohydrates, before the hydrogen peroxide susceptibility assay was performed. As is shown is Table XII, all carbohydrates tried resulted in a similar survival rate after exposure to hydrogen peroxide, except that lactose resulted in about a 10 fold higher survival rate.

To determine if lactose, utilized in medium, was itself able to quench or detoxify hydrogen peroxide, DP1004, was pre-grown in lactose, and resuspended in CATPG before exposure to hydrogen peroxide. As is shown in Table XIII, pneumococcus cultures, pre-grown in glucose, no matter which carbohydrate was used to resuspend cells

before assay, resulted in a similar lower survival rate. Pneumococcus cultures pre-grown in lactose resulted in a similar higher survival rate.

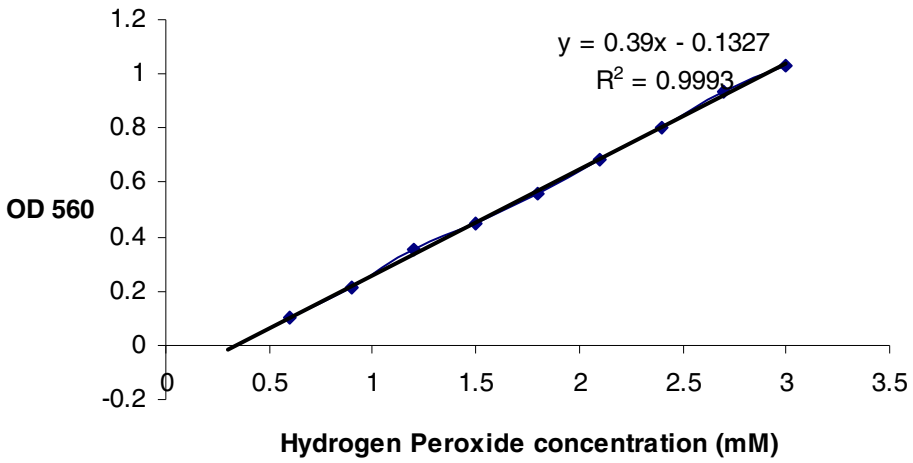


Fig. 20. Standard curve for measurement of hydrogen peroxide production. Hydrogen peroxide samples of known concentrations were used to obtaine a standard curve. In the formula, y denotes for OD_{560}, while x indicates for hydrogen peroxide concentration.

Table X. Effect of the helicase operon on pneumococcal hydrogen peroxide production

Amount of H_2O_2 produced[b], (mM/hr/ 10^8 cfu[a)]	Static culture	Aerated culture
SP1000	0.186 ± 0.020	2.034 ± 0.038
SP1519	0.199 ± 0.023	2.016 ± 0.064

a. The cells were grown in CATP with 22 mM maltose to mid-exponential phase before assay. The data given is an average ± standard deviation of three independent assays.

Table XI. Effect of the helicase operon on transcription of the gene encoding pyruvate oxidase (*spxB*). The cells grown in 22 mM maltose or sucrose to mid-exponential phase were divided into two aliquots. One continued to be grown under static conditions. The other was grown shaking at 200 rpm for 20 minutes at 37°C, following which both were used in assays to measure β -gal units.

Strains	Growth condition[a]	Carbohydrate supplemented	β-galactosidase activity (Miller units)[b]
SP1525	Static	Sucrose	125.2±6.51
SP1525	Static	Maltose	134.3±9.07
SP1525	Aerated	Maltose	237.3±12.12
SP1528	Static	Sucrose	139.4±6.68
SP1528	Static	Maltose	186.4±4.2
SP1528	Aerated	Maltose	301.7±11.71

a. The cells were grown in 22 mM of the indicated sugar. When needed, the cells were aerated by shaking for 60 minutes at 37°C.

b. The data given are averages ± standard deviation derived from at least three independent assays.

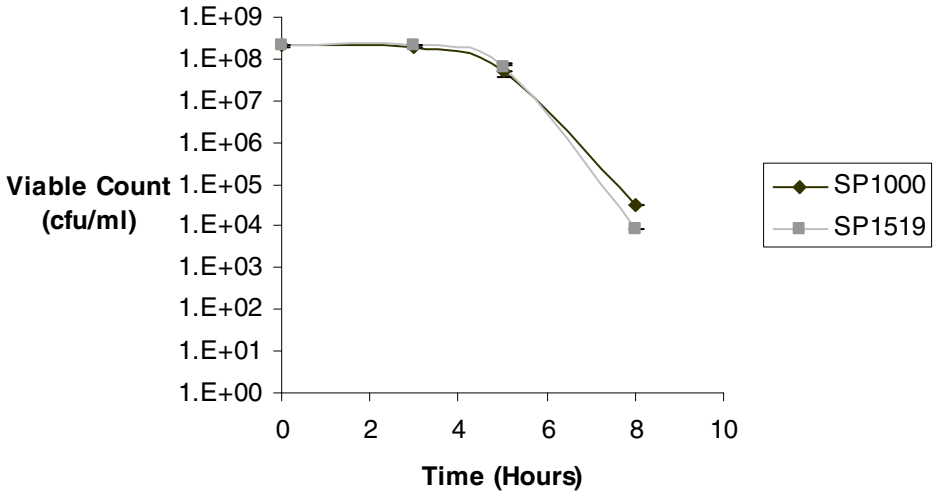


Fig. 21. Effect of the helicase operon on natural autolysis of pneumococcus. Strains SP1000 and SP1519 were grown in CATP with 22 mM maltose before assay. Survival rate was calculated by the viable count obtained at each time point divided by the viable count obtained at time point 0. The error bar represented the standard deviation of results from three independent experiments.

Table XII. Effect of carbohydrate on the survival of pneumococcus in hydrogen peroxide. Strain DP1004 was grown to mid log phase in CATP with 1% (w/v) carbohydrate as listed below before hydrogen peroxide sensitivity assay.

Carbohydrate [a]	Survival rate[b]
Glucose	1.15%±0.74
Lactose	17.65%±2.47
Maltose	1.46%±0.59
Sucrose	1.96%±1.05
Galactose	1.14%±0.59
Fructose	1.36%±0.74
Mannose	1.66%±0.74
Xylose	1.90%±0.93

a. The cells were grown in 1% (w/v) of the indicated carbohydrate.
b. The data given are averages ± standard deviation derived from at least two independent assays.

Table XIII. Effect of lactose on survival of pneumococcus in hydrogen peroxide. Strain DP1004 was grown in CATP with 1% (w/v) carbohydrate listed below, and then pelletted and resuspended in CATP with 1% (w/v) carbohydrate listed below before hydrogen peroxide sensitivity assay.

Carbohydrate used to grow cells	Carbohydrate used to resuspend cells	Survival rate in hydrogen peroxide[a]
Glucose	Glucose	0.83%±0.08
Glucose	Lactose	0.89%±0.11
Lactose	Glucose	24.7%±1.35
Lactose	Lactose	24.1%±1.76

a. The data given are averages ± standard deviation derived from at least two independent assays.

CHAPTER V

Discussions and Conclusion

To date, a variety of conjugative transposons have been discovered and identified among a range of bacterial species. Though there is no commonly accepted explanation on the origin of this class of mobile elements, it has become evident that these elements promote horizontal gene transfer among different species, even between different families of bacteria. Besides the genes encoding the transposition machinery, very often these transposons elements carry genes which are not involved in transposition but often render a certain kind of selective advantage to their host. These genes help to establish a synergitic relationship between the conjugative transposon and host cell, in such a way that they may provide the host cell some type of survival advantage in a specific environment, and this may enable the host cells to out-compete other cells from the same species or different species in a given niche. When host cells gain success, conjugative transposons may be maintained stably in that population.

The first conjugative transposon discovered was associated with the transfer of multiple antibiotic resistance. This discovery, at that time, perfectly explained the emergence and wide distribution of antibiotic resistance in several groups of Gram-positive bacteria. Since then, by tracing the transfer of antibiotic resistance, many

conjugative transposons have been discovered. In recent years, with the completion of genomic sequences from a number of bacterial species, we are identifying a number of conjugative transposons that do not carry any antibiotic resistance genes. This discovery implies that all the known conjugative transposons carrying drug resistance may represent only a small portion of a much larger number of such elements. It also implies that there might be many other types of selective advantages besides antibiotic resistance that such elements may carry and disseminate in the environment. Study of these transposons has already revealed the functions of some of these genes. For example, in *Enterobacteria*, conjugative transposons have been connected with pathogenicity islands which render host species an enhanced ability to produce invasive infections [68]. Also, there have been conjugative transposons identified from soil bacteria in which there are huge operons responsible for the degradation of organic waste in the environment [99]. At the same time, there are still a number of genes residing in conjugative transposons without any known function or biological significance. It is reasonable to believe that some of these genes may have important functions other than antibiotic resistance.

In Tn*5252*, which our lab has been studying for some time, the region carrying chloramphenicol resistance (*cat*) is flanked by direct repeats of an *IS*-like sequence. Due to homologous recombination between the *IS*-sequence flanking the *cat*, *cat* cannot be stably maintained in the absence of selective pressure. This implies that chloramphenicol resistance may not be the most important advantage that pneumococcus acquires from Tn*5252* and there must be other genes that are critical to host cell in the element. The genomic sequence of several bacteria species gives us a powerful tool to further analyze

the non-transfer related regions in Tn*5252*. Almost perfect alignment of the DNA sequence between coordinates 22.5 and 29.9 in Tn*5252* with DNA sequences of transposons in G54, *S. suis* 89/1591, *S. agalactiae NEM316*, *and Streptococcus agalactiae 2603V/R* helped us to narrow our focus on a 7.4 kb operon containing 4 open reading frames. The major part of this operon, ORF 2, was observed to encode a large protein belonging to the DEAH* family.

Since the 7.4 kb helicase operon in the central region of Tn*5252* was also observed in other streptococcal pathogens, we hypothesized that this operon encodes a virulence factor that may enhance pneumococcal virulence during infection. To study the role of the helicase operon during infection, we attempted to introduce the operon from the non-encapsulated laboratory strains into an encapsulated clinical strain by conjugation. Initially filter mating was carried out by filtering cells through a 0.45 μm filter. However, the extra-cellular polysaccharide outside the cell of the clinical strain completely blocked the filter and prevented the solution from passing through. Since filter mating between wild type clinical strains as recipients and laboratory strains carrying the element was not successful, strategies other than filter mating were explored. We hypothesized that the *IS*-like sequence in Tn*5252* also could exist in the chromosome of clinical strains. This hypothesis was supported by results obtained from the Southern hybridization in which SP3001 and SP3101 showed hybridization to probe pSS141. It was most likely due to the homology between an *IS*-like sequence in the chromosome of the clinical strain with an *IS*-like sequence in pSS141. The band pattern representing the

helicase operon was not seen in either SP3001 or SP3101, indicating that the operon was not present in both the "wild type" clinical strains, D39 and WU2.

Though the helicase operon was inserted into the clinical strains by transformation through the mechanism of insertion duplication, it was very unlikely that any gene in the chromosomal DNA of the clinical strains was interrupted due to this event. However, to prevent other complications during insertion of the helicase operon, and to confirm that the differences in capability of infection are solely determined by the helicase operon, we also created strains SP3005 and SP3103 in which the helicase operon was mutated by insertion of cassette carrying an antibiotic resistance determinant.

It has been reported that the colony morphology of a clinical pneumococcal strain determines its capability for infection [91]. Hence, the colony morphology of each strain was examined. All the strains showed "transparent" morphology, based on criteria described previously[91]. Also, it has been reported that phase variation happens with a frequency of 10^{-5} to 10^{-7}. Thus, the possibility that different colony morphology exists in the strains used in the virulence assays can be ruled out.

Results obtained from the virulence assays indicated that the helicase operon would decrease pneumococcal virulence during intraperitoneal challenge, while increasing virulence during intranasal challenge. Though SP3004 cells were able to cause significantly more or less death of mice than SP3001 or SP3005 in intranasal infection and intraperitoneal infection, respectively, in either case, survival rate of mice obtained

from SP3001 was not significantly different from strain SP3005. Similar results were also observed with SP3101 and SP3103. This result helped to rule out the possibility that insertion of the helicase operon into the clinical strains affected any virulence-related gene(s) in the host cell DNA. It also confirmed that it was the helicase operon that was responsible for the difference in infection. It also implied that the helicase operon was active in both types of infection, since in each group the, "wild type" with the operon showed a significant differently survival rate compared to "wild type" cells and "wild type" cells with the mutated operon.

If the helicase operon is able to render some advantage or disadvantage during pneumococcal infection as indicated in virulence assays, in a mixed infection with two strains, the strain with the helicase operon should outgrow the "wild type" during intranasal infection, or would be outcompeted by the "wild type" during intraperitoneal infection. For this reason, competition experiments were performed to infect mice with mixed pairs of strains, SP3003 and SP3004. Since it has been reported that clinical strains with different chromosomal markers could result in a different capability to survival in host, SP3001 and SP3003 were mixed and used for infection as a control. Consistent with a previous report, SP3001 (*str*) outnumbered SP3003 (*nov*) during the nasopharyngeal infection. Above that, SP3004 showed about 5 times better survival over SP3001 in the nasopharyngeal region. On the other hand, SP3001 was outnumbered by SP3003 during intraperitoneal infection. Above that, SP3004 was at least 4 times worse than SP3001 in surviving during intraperitoneal infection. Consistent with this, similar results were also obtained with WU2 derivatives. All of these confirmed the conclusion we drew in the

virulence assay. Sequence analysis based on deduced amino acid sequences suggested cytoplasmic localization of the proteins deduced by ORF1, 2 and 3. Results from competition experiment also implied that the function of the helicase was probably within the cell since a secreted factor would be expected to help both strains during mixed infection and would not lead to a significant difference in their capability of survive in the host.

Though there was evidence that the helicase was active during infection, by inserting a reporter gene into ORF2, the *lacZ* fusion experiments showed that it was not active when grown under laboratory conditions. To further determine the conditions required for the induction of helicase operon, SP1472 was plated out with X-gal in a chemically defined medium with various supplements. At least 5 μ mol Fe^{2+} was required for induction of the helicase operon; however, no significant induction was observed in the chemically defined medium broth with sufficient Fe^{2+} supplemented. This was probably because the fact that Fe^{2+} is only one of several factors required for induction. Without the presence of other required factors, there would not be full induction.

Based on the plating results, we conclude that Fe^{2+} is required for the induction of the helicase operon. Sequence analyses predicted a metal binding motif and helix-turn-helix motif was predicted in ORF1. We hypothesize that the protein encoded by ORF1 is involved in the regulation of the helicase operon expression. To test this hypothesis, SP1516 and SP1517 were created and plated out on a chemically defined medium with X-gal and Fe^{2+} supplemented; both strains gave a light blue color after 72 hours. This

result ruled out the possibility that ORF1 was the positive regulator required for expression of the helicase operon since there was successful induction without ORF1. Also, this result ruled out the possibility that ORF1 functioned as a negative regulator, since SP1516 did not show any increased induction compared to SP1517. Taken together, these results make it very unlikely that ORF1 is involved in regulation of the helicase operon expression.

Though Fe^{2+} seemed to weakly induce the helicase operon, the conditions for full induction of the operon remained unknown. However, to study its function, we inserted an inducible promoter in between ORF1 and the natural promoter of the helicase operon so that the operon could be induced. Insertion of the P_{malX} promoter, which would be induced by maltose supplemented in medium, was achieved by cross-over PCR followed by transformation of DNA into pneumococcus. The newly created strain, SP1460, was analyzed by Southern hybridization to confirm that the construct in the promoter region was as expected. Also, by insertion of a reporter gene into ORF2, it was confirmed that the helicase operon could be induced by maltose as expected. At the same time, some leaky expression in the presence of glucose was also observed.

Our attempts to identify all the genes influenced by the induction of the helicase operon were not quite successful. One possible reason was that the leaky expression of P_{malX} in the presence of glucose interfered in the screening procedure employed. Also it is possible that our method for random insertion of a reporter gene into the chromosomal DNA did not guarantee insertion into every possible gene in the chromosome.

Our results indicated that induction of the helicase operon required at least 5 μ mol of Fe^{2+} which has been reported to react with hydrogen peroxide in the Fenton reaction and generate hydroxyl radicals, the active agent causing damage to cells[98]. As previously reported, pneumococcus generate hydrogen peroxide as a metabolic by-product during aerobic growth[14]. These observations led us to wonder whether the helicase operon affected pneumococcal sensitivity to hydrogen peroxide. Our results indicated that the helicase operon made pneumococcus cells more sensitive to hydrogen peroxide which seemed to imply two possible targets of the helicase operon: superoxide dismutase (*sodA*)[98], which would detoxify hydroxyl radicals generated during the Fenton reaction; and pyruvate oxidase (*spxB*)[15] which would catalyze a reaction to generate hydrogen peroxide. The first possibility could be ruled out since pneumococcus did not show increased sensitivity to the other oxidative agents, such as methyl viologen and tert-butyl hydroperoxide which produce superoxide anions. The superoxide anions are known to be scavenged by superoxide dismutases[98].

A previously reported model to explain pneumococcal sensitivity to hydrogen peroxide was that the ATP level in cells determines the sensitivity to hydrogen peroxide[15]. Increased activity of pyruvate oxidase would in turn lead to more ATP production and more hydrogen peroxide production which would lead to more resistance to exogenous hydrogen peroxide. Results we obtained did not quite fit with this model. In our system, when the expression of the helicase operon was induced, we observed increased transcription of *spxB* with increased sensitivity to hydrogen peroxide. However,

no significant change in hydrogen peroxide production was observed. One possible explanation would be that when the helicase operon was induced, expression of genes other than pyruvate oxidase were also affected, which led to more consumption of ATP and hence more sensitivity to hydrogen peroxide. It is also possible that sensitivity to hydrogen peroxide is much more complicated than determined solely by ATP levels and that some other factors may play an important role in this process.

It is possible that the helicase operon increases virulence of pneumococcus during intranasal infection by directly up-regulating or enhancing the expression of specific virulence factors. However, this does not explain why the virulence of pneumococcus during intrperitoneal infection was attenuated. From our results, we infer that when the expression of the helicase operon was induced, multiple phenotype changes take place. This implies that there are systematic changes in gene expressions rather than a change in a few specific genes.

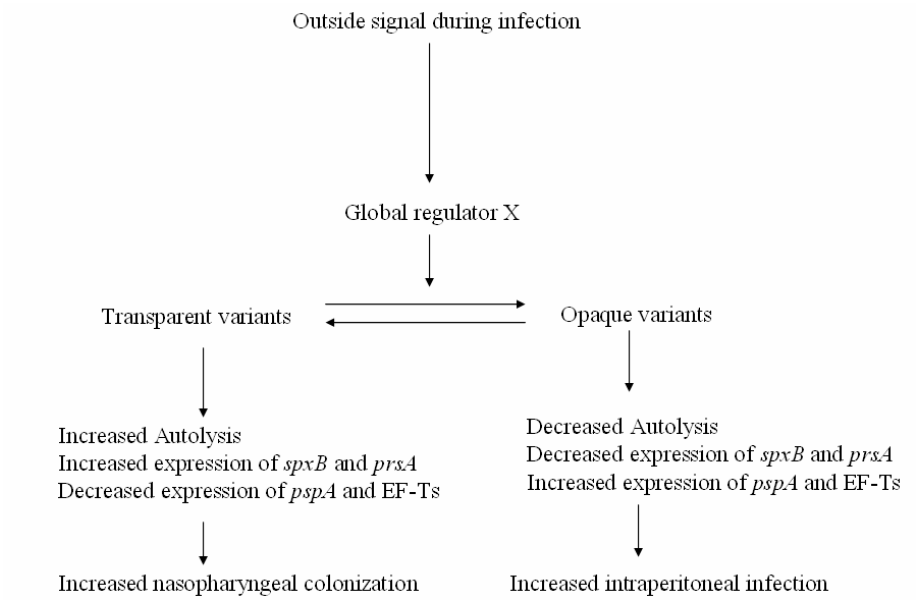
Based on all these results, the following model is proposed to explain the mechanism of how the helicase operon modulates the virulence of pneumococcus. As shown in Fig 22, in pneumococcal cells, there exists a global regulator X which is involved in regulation of phase changes between transparent variants and opaque variants. During pneumococcal infection, unknown signals from the host during infection are capable of influencing the expression of X to make a complete change between transparent variants and opaque variants. While expression of the helicase operon only slightly changes the expression of X, it always results in a slight phase shift toward

transparent phase. During intranasal infection, the slight shift toward the transparent phase will make cells “more transparent” which will lead to enhanced expression of a series genes involved in colonization in the nasopharyngeal region. During intraperitoneal infection, a slight shift toward the transparent phase will make cells “less opaque” which will lead to slightly attenuated expression of a series of genes involved in invasive infection. Consistent with this hypothesis, when grown under laboratory conditions, the strain with an induced helicase operon showed an increase in the transcription of gene *spxB* and enhanced autolysis both of which fit with characteristics of a transparent variant.

As shown in Fig. 22, it was reported that compared to opaque variants, transparent variants have increased expression in *prsA* (spr0844) which is involved in protein maturation, decreased expression in elongation factor (spr2019) [47] and decreased expression of pspA [100]. To further examine the hypothesis above, experiments can be proposed to determine if there is an increase of expression of *prsA* and decreased expression of elongation factor and *pspA* in strains with an induced helicase operon. Even further, in an encapsulated background, experiments can be proposed to determine if there is a decreased amount of capsule in strains with the induced helicase operon.

Phase variation is a strategy that pneumococcus have developed to adapt to different environments *in vivo*. Though pneumococcus is able to cause infection in quite different places, the nasopharyngeal region is the place it has to colonize first. Since transparent variants are more efficient in colonizing in the nasopharyngeal region while

A.



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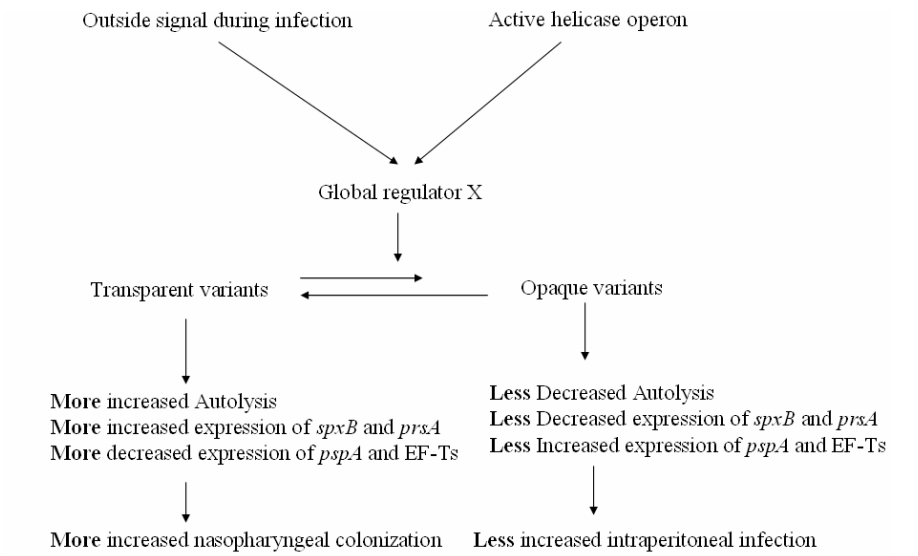

Fig. 22. Hypothesis to explain the mechanism of how the helicase operon influences the virulence of pneumococcus. A. In the strain without the helicase operon B. In the strain with an induced helicase operon

opaque variants are more efficient in causing intraperitoneal infections, it is expected that before further invasive infection, there must be a phenotypic change to make pneumococcus cells adapt the environment change. Efforts to understand the details of pneumococcus phase variation have not been successful so far, since this change happens at quite a low frequency under laboratory conditions. If our hypothesis on the mechanism of how the helicase operon modulates the virulence is proved to be correct, it will provide an alternative way to tackle the problem of phase variation. Further work to identify the targets of the helicase operon may help to uncover the machinery involved in regulation of phase variation and gain more understanding on the pathogenesis of pneumococcus.

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VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: MODULATION OF VIRULENCE OF *STREPTOCOCCUS PNEUMONIAE* BY AN OPERON IN CONJUGATIVE TRANSPOSON TN*5252*

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Title of Study: MODULATION OF VIRULENCE OF *STREPTOCOCCUS PNEUMONIAE* BY AN OPERON IN CONJUGATIVE TRANSPOSON TN*5252*

Pages in Study: 130 Candidate for the Degree of Doctor of Philosophy

Major Field: Microbiology

Scope and Method of Study: The aim of this work was to study of the function of the 7.4 kb DNA in the central region of Tn*5252*. Through virulence assay, we would determine if this operon is involved in the pathogenesis of *S. pneumoniae*. Furthermore, if this operon was involved in pathogenesis, we would carry out experiments to determine how this operon is involved in the pathogenesis of *S. pneumoniae*.

Findings and Conclusions: The streptococcal mobile element, Tn*5252* (47 kb), carries an 8 kb operon containing four genes the largest of which is about 6 kb and highly homologous to eukaryotic SNF2-like DNA methyl transferase/helicases that are involved in gene regulation. The helicase operon was introduced into the chromosome of clinical pneumococcal strains by additive transformation. When introduced intraperitoneally into young female BALB/c mice, strains bearing the intact helicase operon were found to be significantly less pathogenic than the parental wild type strain or the one with the mutated operon. However, when introduced into the nasopharyngeal region, cells carrying the intact helicase operon were able to cause significantly more death than the other two. In competition experiments, the cells carrying the helicase operon were much more successful than others in colonization of the nasopharyngeal niche while no differences in growth patterns between the two were found when cocultured *in vitro*. *In vitro* the expression of the helicase operon was not constitutive. Following placement of the inducible pneumococcal *malX* promoter upstream of the operon, it was observed that activation of the operon by maltose renders the cells more susceptible to oxidative damage by hydrogen peroxide Also it was observed that activation of the operon lead to induced expression of *spxB*, and increased autolysis. Taken together with the results virulence assay, it is suggested that activation of the helicase operon would have an effect on pneumococcal phase variation.

ADVISER'S APPROVAL: Dr. Moses Vijayakumar