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The Cgl1281-encoding putative transporter of the cation diffusion facilitator family is responsible for alkali-tolerance in *Corynebacterium glutamicum*

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Abstract

Mutants of *Corynebacterium glutamicum* that were unable to grow under mild alkaline pH conditions were isolated by mutagenesis. Strain AL-43 exhibiting the highest sensitivity to alkaline pH among the mutants was selected and used to clone a DNA fragment that could complement the phenotype. Sequencing and subcloning of the cloned 4.0-kb *Eco*RI DNA fragment showed that the Cgl1281 gene was responsible for the complementation. The deduced amino acid sequence of Cgl1281 was found to show significant sequence similarity with CzcD, a $Me^{2+}/H^{+}(K^{+})$ antiporter, from *Bacillus subtilis* and also possess the features of the cation diffusion facilitator (CDF) family: the presence of 6 putative transmembrane segments and a signature sequence, indicating that the gene product is a member of the CDF family. Chromosomal disruption of the Cgl1281 rendered *C. glutamicum* cells sensitive to alkaline pH as well as cobalt, while expression of the gene from a plasmid restored alkali-tolerance to the wild-type level and also led to increased cobalt resistance. These results demonstrated that the putative transporter of the CDF family mediates resistance to cobalt and also plays a physiological role in alkaline pH tolerance in *C. glutamicum*.

Keywords Alkaline pH tolerance · Cation diffusion facilitator family · Cobalt resistance · *Corynebacterium glutamicum*

Introduction

Corynebacterium glutamicum is an industrially important microorganism that is

widely used for the production of various amino acids (Kinoshita and Nakayama 1978). In the industrial fermentation, *C. glutamicum* cells must adapt to various suboptimal conditions due to the considerable heterogeneity within large-scale fermentors. To meet these demands, the cells, like other organisms, are assumed to possess a variety of mechanisms for adaptation. One such mechanism is pH homeostasis that acidifies or alkalinizes the cytoplasm relative to the external environment. Actually, *C. glutamicum* is adapted to relatively high alkaline conditions (Barriuso-Iglesias et al. 2006). However, there has been no study on the mechanism of pH homeostasis of this bacterium despite its basic and industrial interest.

Neutralophilic bacteria such as *Escherichia coli* and *Bacillus subtilis* generally grow at a relatively broad range of external pH values of 5.5-9.0, and maintain a cytoplasmic pH within a narrow range of 7.4-7.8 (Padan et al. 2005). The machinery responsible for alkaline pH homeostasis consists of many cellular factors, and their intricate interplay is assumed to support maintenance of a near neutral cytoplasmic pH under the alkaline conditions (Padan et al. 2005). Among the machinery, monovalent cation/proton antiporters are often used for alkaline pH homeostasis in bacteria (Padan et al. 2005). The best studied example is Na^+/H^+ antiporter NhaA, which is a representative antiporter responsible for both Na^+- and alkaline pH homeostasis in *E. coli* (Goldberg et al. 1987; Karpel et al. 1988; Padan et al. 2005).

It is noteworthy that other antiporters than the monovalent cation/proton specific antiporters are involved in alkaline pH homeostasis as well. Transpositional insertion libraries of *B. subtilis* for alkali-sensitive mutants led to the identification

of Tet(L), a multifunctional tetracycline-metal/ H^+ antiporter, with a role in alkaline pH homeostasis (Cheng et al. 1994). Also in *E. coli*, it was recently reported that MdfA, a multidrug-resistance transporter, also catalyzes Na^+- or K^+-dependent proton transport, in addition to its original drug/proton antiport function, and has additional function of alkaline pH homeostasis (Lewinson et al. 2004).

Our laboratories have recently determined the whole genome sequence of the representative wild-type strain of *C. glutamicum,* ATCC 13032 (Ikeda and Nakagawa 2003). The genomic data (accession number: BA000036) have provided us with a flood of information about putative transport proteins including antiporters (Winnen et al. 2005). However, none of those have been examined for their functions from the viewpoint of pH homeostasis. Furthermore, a large part of the putative transporters remain to be assigned even a tentative function. To identify a major player in alkaline pH tolerance of this bacterium, we generated mutant libraries with more or less alkali-sensitive phenotypes and selected the best mutant with a distinctive alkali-sensitive phenotype. By using this strain as a host, we succeeded in cloning the gene that could complement the phenotype. The sequence of the gene and the primary structure of the gene product revealed that it is the Cgl1281-encoding putative transporter of the cation diffusion facilitator (CDF) family that occurs in both prokaryotes and eukaryotes (Paulsen and Saier 1997). This paper shows critical involvement of the CDF protein in alkaline pH tolerance in *C. glutamicum*.

Materials and methods

Bacterial strains and plasmids

The wild-type strain of *C. glutamicum* used in this study is strain WT-96, a single-colony derivative of *C. glutamicum* ATCC 31833 (Kinoshita 1999). *E. coli* DH5α (Sambrook and Russell 2001) was used as a host for cloning of the PCR products. Plasmid pCS299P (Mitsuhashi et al. 2004), a *C. glutamicum*-*E. coli* shuttle vector, was used to construct a genomic library and also to clone the PCR products. Plasmid pESB30, which is nonreplicative in *C. glutamicum*, is a vector for gene replacement in *C. glutamicum* (Mitsuhashi et al. 2004). Plasmid pCgl1281d that contained the internally deleted Cgl1281 gene in vector pESB30 was used to replace the wild-type chromosomal gene by the deleted gene.

Media and culture conditions

Complete medium BY, minimal medium MM, and enriched minimal medium MMYE (Katsumata et al. 1984; Takeno et al. 2006) were used for growth of *C. glutamicum* strains. Low-salt complex medium LS (10g/liter tryptone, and 5g/liter yeast extract) was used to examine the alkaline pH tolerance of cells with and without the Cgl1281 gene. Media at different alkaline pH conditions were prepared using 100 mM CAPS buffer for MM and MMYE media and using 70 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane (Bistris propane) buffer for LS medium as described previously (Lewinson et al. 2004; Kosono et al. 2005). For growth at acidic pH conditions, MM medium was prepared using 100 mM MES buffer. Agar plates were made by the addition of Bacto-Agar (Difco) to 1.6%. For

liquid culture of *C. glutamicum* strains, a 0.05-ml amount of the seed culture grown aerobically at 30ºC in BY medium for 12 h was inoculated into 5 ml of medium in a L-type test tube and cultivated at 30ºC using a Monod shaker (Taitec Inc., Saitama, Japan) at 40 strokes/min. Co^{2+} resistance was examined in MMYE medium at pH 7.5. When required, kanamycin was added at the final concentration of 20 μg/ml.

Mutagenesis

Cells of strain WT-96 grown in 3 ml of BY medium were suspended in 3 ml of 50 mM Tris-maleate buffer (pH 6.0) containing *N*-methyl-*N*´-nitro-*N*-nitrosoguanidine (400 μg/ml), and incubated at 30ºC for 30 min. The mutagenized cells were harvested, washed twice in 3 ml of the above buffer, and their appropriate dilutions were spread on BY agar plates (pH 7.0), which were then used to screen for alkali-sensitive mutants by replica plating.

Recombinant DNA techniques

Standard protocol (Sambrook and Russell 2001) was used for the construction, purification and analysis of plasmid DNA, and transformation of *E. coli*. Chromosomal DNA was extracted from protoplasts of *C. glutamicum* by the method of Saito and Miura (1963). The protoplasts were prepared by the method of Katsumata et al. (1984). Transformation of *C. glutamicum* by electroporation was carried out by the method of Rest et al. (1999), using Gene pulser and Pulse controller (BioRad, USA). PCR was performed with DNA thermal cycler GeneAmp

PCR System 9700 (Applied Biosystems, CA, USA) using TaKaRa LA-Taq™ (Takara Bio, Shiga, Japan). PCR products were purified using GENECLEAN III KIT (Qbiogene, CA, USA).

Cloning and subcloning of the Cgl1281 gene

Chromosomal DNA from *C. glutamicum* wild-type strain WT-96 and vector pCS299P were partially digested with *Eco*RI and ligated. The genomic library was used to transform the protoplasts of an alkali-sensitive mutant AL-43. Transformants were selected on MM agar plates (pH 9.5) containing kanamycin. For subcloning of the Cgl1281 gene, plasmid pCgl1281 was constructed as follows. The region containing the Cgl1281 gene was amplified by PCR using primers pr1 and pr2 with pEco4.0 as a template (Table 1). The reaction was performed as follows: heating to 94°C for 1 min; 25 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 2 min; followed by extension for 6 min at 72°C. The forward primer pr1 was designed to anneal at positions -112 to -89 with the Cgl1281 gene, whereas the reverse primer pr2 annealed at the positions on vector pCSEK20. The resulting 1.9-kb fragment was digested with *Eco*RI, and then ligated to the *Eco*RI-digested pCSEK20 to yield pCgl1281.

Generation of the Cgl1281-disrupted mutant

Plasmid pCgl1281d containing the internally deleted Cgl1281 gene was constructed as follows, and was used to replace the wild-type chromosomal gene with the

deleted gene. Primers used in this study are listed in Table 1. The 5'-region of the Cgl1281 gene was amplified by PCR using primers pr3 and pr4 with the wild-type genomic DNA as a template. Similarly, the 3'-region of the gene was amplified using primers pr5 and pr6. Each of the reactions was performed with the following programs: heating to 94°C for 5 min; 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; followed by extension for 3 min at 72°C, which amplifies the 800-bp fragments. As the two primers pr4 and pr5 were complementary to each other, fusion PCR was performed using the purified 5'-region-Cgl1281 and 3'-region-Cgl1281 fragments as templates and the primers pr3 and pr6 with the following program: heating to 94°C for 5 min; 25 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; followed by extension for 3 min at 72°C. The resulting 1.6-kb fragment contained the deleted Cgl1281 gene which was shortened from 957 bp to 120 bp by in-frame deletion of the inner sequence. The fragment was digested by *Bgl*II and then ligated to *Bam*HI-digested pESB30 to yield pCgl1281d. Defined chromosomal deletion of the Cgl1281 gene was accomplished using the pCgl1281d, via two recombination events as described previously (Ohnishi et al. 2002). A strain carrying the Cgl1281 deletion in the wild-type background was designated strain ΔCgl1281.

Results

Screening for alkali-sensitive mutants

We first examined the pH tolerance of *C. glutamicum* wild-type strain WT-96 on

MM agar plates adjusted at different pH values using buffers. As shown in Fig.1, strain WT-96 grew over a relatively broad range of pH 6.0-10.0. Then, mutagenized cell libraries of strain WT-96 were screened for alkali-sensitive mutants by replica plating to MM agar plates at pH 7.0 and pH 9.5. More than 100 candidates were repeatedly examined for their alkali-sensitive phenotypes on the buffered plates and finally four typical alkali-sensitive mutants were selected. The four mutants, designated AL-40, AL-42, AL-43, and AL-77, exhibited different alkali-sensitivities whereas they all showed similar growth to the wild type at neutral and acidic pH conditions (Fig. 1).

Identification of the gene that complements the alkali-sensitive phenotype

Among the four alkali-sensitive mutants, mutant AL-43 was used as a first priority to clone the gene that could complement the phenotype because it showed the highest alkali-sensitivity and thus was assumed to be impaired in the primary function responsible for alkaline pH homeostasis. By employing the shotgun method of cloning, we obtained a plasmid carrying a 4.0-kb *Eco*RI DNA fragment of *C. glutamicum* chromosomal DNA that enabled the mutant cells to grow at pH 9.5. The plasmid was designated plasmid pEco4.0. The nucleotide sequence of the cloned fragment in plasmid pEco4.0 revealed the presence of four intact genes, Cgl1278, Cgl1279, Cgl1280, and Cgl1281 (Fig. 2A and B). Genome databases for this organism identified the last Cgl1281 gene as encoding a putative Co/Zn/Cd efflux transporter, whereas the former three genes remained hypothetical proteins. Based on the gene map of the fragment, we subcloned the internal regions and examined

their ability to complement the alkali-sensitive phenotype of mutant AL-43. As a result, plasmid pCgl1281 containing only the intact Cgl1281 gene (Fig. 2C) was found to complement the alkali-sensitive phenotype, indicating that the Cgl1281 gene was responsible for the complementation.

Characteristics of the primary structure of the Cgl1281 gene product

The Cgl1281 gene product encodes a protein of 318 amino acid residues with a predicted molecular mass of 34,023 Da. As is evident from the alignment shown in Fig. 3A, the amino acid sequence showed high homology (34% identity) to the CzcD protein (accession number: CAB14606) of *B. subtilis* 168. The *B. subtilis* CzcD, a member of the CDF family, is an antiporter that effluxes divalent cations, including Co^{2+}, in exchange for H^+ and K^+ (Guffanti et al. 2002; Wei et al. 2006). Therefore, the Cgl1281 gene product was assumed to be a CzcD-like CDF protein relevant to divalent cations efflux. Since the members of this CDF family have been reported to possess six putative transmembrane spanners with a signature sequence that begins with a conserved serine residue (Paulsen and Saier 1997), we examined whether these two features occurred in the Cgl1281 gene product. As shown in Fig. 3A, the CDF family-specific signature sequence was observed in the Cgl1281 gene product. In addition, hydropathy analysis indicated that the Cgl1281 gene product contains six putative transmembrane spanners, like the *B. subtilis* CzcD (Fig. 3B). From these observations, we concluded that the Cgl1281 gene product is a member of the CDF family.

Cgl1281-mediated resistance to cobalt

If the Cgl1281 gene product was indeed a *B. subtilis* CzcD-like CDF protein that effluxes toxic Co^{2+} in exchange for H^+ and K^+ (Guffanti et al. 2002; Wei et al. 2006), it was predicted that the presence of Cgl1281 would render *C. glutamicum* cells Co^{2+} resistant. To examine this possibility, we constructed a wild-type derivative with an in-frame deletion in the internal region of Cgl1281 (Fig. 2D), and compared Co^{2+} resistance among the wild-type strain, its Cgl1281-disrupted strain ΔCgl1281, and strain ΔCgl1281 overexpressing the gene from plasmid pCgl1281 by using both agar plates and liquid medium at pH 7.5. As shown in Fig. 4A and B, the resistance of strain ΔCgl1281 carrying the empty vector to Co^{2+} was lower than that of its isogenic wild-type parental strain carrying the same vector, whereas strain ΔCgl1281 carrying pCgl1281 showed higher resistance to Co^{2+} than the wild-type strain carrying the vector. Although the differences were not prominent, we confirmed by three independent experiments that the Cgl1281-dependent resistance to Co^{2+} was reproducible and significant. To further verify the results, we conducted the same experiments at a neutral pH and also at a more alkaline pH (pH 8.0), which resulted in substantially the same results as those at pH 7.5 (data not shown). These results demonstrate that the Cgl1281 gene product mediates Co^{2+} resistance in this organism, supporting the *B. subtilis* CzcD-like, Co^{2+}-eflluxing function of the protein.

Cgl1281-mediated alkali-tolerance

Studies on functional characterization of machinery relevant to alkaline pH homeostasis are often hampered by redundant transport systems. To minimize the interference of other systems, previous studies often use low-salt complex media, such as a modified Luria-Bertani medium, for elucidating the functions of individual systems (Cheng et al. 1994; Lewinson et al. 2004; Kosono et al. 2005; Liew et al. 2007). Therefore, we used a low-salt complex medium (LS medium) reported by Lewinson et al. (2004) to evaluate the Cgl1281-mediated alkali-tolerance. Only magnesium chloride was supplemented to the low-salt medium at 10 mM because preliminary growth experiment showed somewhat a stimulatory effect on the growth of *C. glutamicum* cells at a neutral pH. Under the conditions, the growth of the wild-type strain carrying the empty vector, the Cgl1281-disrupted strain ΔCgl1281 carrying the same vector, and strain ΔCgl1281 carrying plasmid pCgl1281 were studied at various pHs both on agar plates and in liquid medium. On agar plates (Fig. 5A), the wild-type strain grew somewhat better than strain ΔCgl1281 even at a neutral pH. As the pH of the medium increased, the negative effect of the chromosomal deletion of Cgl1281 became obvious. Under the same conditions, the expression of Cgl1281 on a plasmid rendered strain ΔCgl1281 alkali-tolerant, like the wild-type strain. The advantageous effect of the Cgl1281 expression at elevated pHs was also evident in liquid medium (Fig. 5B). These results indicate that the Cgl1281 gene product plays a physiological role in alkaline pH tolerance in *C. glutamicum*.

Discussion

In this study, we identified a protein responsible for alkaline pH homeostasis for the first time in industrially important *C. glutamicum*. The identified protein, the Cgl1281 gene product, had significant sequence similarity with the CzcD protein from *B. subtilis*, and a direct correlation between the presence of the Cgl1281 gene and the Co^{2+} sensitivity was shown. From these results, we conclude that the Cgl1281 gene encodes a Co^{2+}-efflux transporter which also plays a physiological role in alkaline pH homeostasis in *C. glutamicum*. Interestingly, the protein was found to belong to the CDF family by hydropathy and sequence analyses. As far as we know, involvement of the CDF proteins in alkaline pH homeostasis has not been reported in bacteria. The *C. glutamicum* genome encodes three other homologues (Cgl2072, Cgl2133, and Cgl2783) of *B. subtilis* CzcD although the similarities with *B. subtilis* CzcD are lower than the case of the Cgl1281 gene product. Any of these gene products remains to be functionally characterized, and it will be of interest to ascertain whether those homologues have a similar function in alkaline pH homeostasis.

E. coli Na^+/H^+ antiporters NhaA and NhaB belong to the monovalent cation/proton antiporter superfamily (CPA) (Chang et al. 2004). As exemplified by those antiporters, many CPA-type Na^+/H^+ antiporters have been demonstrated to participate in alkaline pH homeostasis in bacteria (Padan et al. 2005). However, do the CPA proteins always play a dominant role in alkaline pH tolerance? So far, functional complementation screens using *E. coli* Na^+ /H^+ antiporter-disrupted strains with a Na^+-sensitive phenotype have become a common route to clone new antiporter genes. Actually, those *E. coli* mutants have been extensively used to identify Na^+ /H^+ antiporter genes from diverse bacteria (Hiramatsu et al. 1998;

Padan et al. 2001; Yang et al. 2005; Kurz et al. 2006). Even if other Na^+-independent transporters such as the CDF proteins are involved in alkaline pH homeostasis, such transporters cannot be obtained by the previous screens probably because of their inability to complement the Na^+-sensitive phenotype of the recipient *E. coli* mutants. That would be the reason why Na^+/H^+ antiporters have so far been considered as the typical machinery for alkaline pH homeostasis in bacteria. In this study, we identified a functionally different CDF protein in *C. glutamicum*. This might be due to our approach of screens based on direct complementation of an alkali-sensitive phenotype. Considering our results, it is worth to study the involvement of the CDF proteins in alkaline pH homeostasis of individual bacterial strains.

Recently, Lewinson et al. (2004) have identified unique machinery, MdfA, for alkaline pH homeostasis in *E. coli* and reported an extraordinary effect of MdfA on alkali-tolerance. According to their data, the *mdfA*-deleted *E. coli* strain exhibited marginal growth at pH 8.75 on low-salt LB-agar plates supplemented with Na^+ or K^+, but the upper limit of pH was broadened to 9.25 by plasmid-mediated expression of the *mdfA* gene in the host. This indicates that the MdfA protein would expand the pH range by 0.5 units. Compared with this case, Cgl1281 likely has a relatively large capacity for alkali-tolerance because Cgl1281 expanded the pH range by almost 1.0 unit under similar culture conditions; the expression of Cgl1281 extended the pH tolerance from around pH 8.75 up to around 9.75 (Fig. 5A). These results strongly suggest that Cgl1281 plays a major role in alkaline pH tolerance of *C. glutamicum*.

Previous information about the properties of proteins responsible for

alkaline pH homeostasis in bacteria has shown that those systems generally depend on Na^+ or K^+ (Padan et al. 2005). This is not confined to many CPA-type $Na^+(K^+)/H^+$ antiporters but is true of other systems such as the multifunctional *E. coli* MdfA (Lewinson et al. 2004) and *B. subtilis* Tet(L) (Cheng et al. 1994). Therefore, disruption of the systems in individual strains has usually resulted in both Na^+- and alkali-sensitive phenotypes (Padan et al. 1989, 2005; Cheng et al. 1996). In contrast, in our case, the Cgl1281-disrupted mutant showed an alkali-sensitive phenotype, but not a Na^+-sensitive phenotype (data not shown). Considering this observation as well as the finding that Cgl1281 showed a significant sequence similarity with *B. subtilis* CzcD, a $Me^{2+}/H^+(K^+)$ antiporter, it seems reasonable to propose that Cgl1281 effluxes a certain divalent cation in exchange for H^+, thereby acidifying the cytoplasm relative to the external environment. As the possible metal substrates for the protein, divalent cations such as Mg^{2+}, Fe^{2+}, and Mn^{2+}, rather than Co^{2+}, could be the candidates because the culture media used were not supplemented with Co^{2+} while the other divalent cations are usually contained in considerable amounts. To define cytoplasmic metal substrates for the protein, we need to study the Cgl1281-mediated antiport reactions in an *in vitro* system. At present, such study is hindered by difficulty of the preparation of the inverted membrane vesicles from *C. glutamicum*, the natural host, and thus its improvement will be the priority toward the next step.

In this study, we have focused on the Cgl1281 gene that complemented the alkali-sensitive phenotype of mutant AL-43 because the mutant exhibited the highest sensitivity to alkaline pH among the alkali-sensitive mutants obtained. However, in the similar shotgun cloning experiments using other typical

alkali-sensitive mutants, AL-40, AL-42, and AL-77 (Fig. 1), we have obtained different DNA fragments, which contain the genes annotated as an ABC-type transporter, a permease of the major facilitator superfamily, and a divalent heavy-metal cation transporter, respectively. Although functional characterization of each gene is yet to be done, we speculate that their interplay allows growth over a broad range of external pH values under different cation conditions in this organism. Elucidation of other players in alkaline pH tolerance of this industrially important bacterium will be an extension of this study, which would lead to a new possibility of creation of industrially more useful strains with robust pH homeostasis.

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Figure legends

Fig. 1 Alkali-sensitivities of four typical mutants. Appropriate dilutions (ca. 10^4/ml) of cultures of *C. glutamicum* wild-type strain WT-96 and its alkali-sensitive mutants were spotted onto MM agar plates adjusted at indicated pH values. Growth was assessed after two days incubation at 30°C.

Fig. 2 Schematic representation of subcloning and disruption of the Cgl1281 gene. (A) Genetic organization of the DNA region around the Cgl1281 gene. (B) The originally cloned 4.0-kb *Eco*RI fragment in vector pCS299P. (C) The subcloned 1.9-kb fragment in vector pCSEK20. (D) The 1.6-kb fragment containing the deleted Cgl1281 gene in vector pESB30. Primer sequences are described in Table 1.

Fig. 3 (A) Sequence alignment of the deduced amino acid sequence of the Cgl1281 gene product with CzcD from *B. subtilis*. The amino-acid residues conserved in both sequences are highlighted in *black*. The CDF family-specific signature region is *boxed*. The signature sequence is:

SX(ASG)(LIVMT)$_2$(SAT)(DA)(SGAL)(LIVFYA)(HDN)X$_3$DX$_2$(AS) [X means any residues; alternative residues at any one position are in parentheses.] (Paulsen and Saier 1997) The serine and aspartate residues strictly conserved among the CDF family proteins are indicated by *asterisks*. (B) Comparison of the hydropathy plot of the Cgl1281 gene product with CzcD from *B. subtilis*. Hydropathy values were calculated by the method of Kyte and Doolittle (1982) with a sliding window of 20 residues. The values were plotted from the N terminus to the C terminus. The six peaks (I-VI) correspond to the six putative transmembrane spanners.

Fig. 4 Cobalt resistance of Cgl1281-disrupted and -overexpressed strains. (A) Serial dilutions (1/10 each) of cultures of strains ΔCgl1281 carrying vector pCSEK20, ΔCgl1281 carrying plasmid pCgl1281, and wild-type WT-96 carrying vector pCSEK20 were spotted onto MMYE agar plates (pH 7.5) with increasing $CoCl_2$ concentrations. Growth was assessed after two days incubation at 30ºC. (B) Strains ΔCgl1281 carrying vector pCSEK20 (*circles*), ΔCgl1281 carrying plasmid pCgl1281 (*squares*), and wild-type WT-96 carrying vector pCSEK20 (*triangles*) were grown in liquid MMYE medium (pH 7.5) with increasing $CoCl_2$ concentrations at 30ºC under aerobic conditions. Growth at indicated $CoCl_2$ concentrations is shown relative to the growth rate (doubling /hour) in the absence of $CoCl_2$ in each strain. The growth rates of the three strains in the absence of $CoCl_2$ were almost identical (0.46-0.47). Error bars indicate standard deviations from three independent experiments.

Fig. 5 Alkali-sensitivities of Cgl1281-disrupted and -overexpressed strains. (A) Appropriate dilutions (ca. 10^4/ml) of cultures of strains ΔCgl1281 carrying vector

pCSEK20, ΔCgl1281 carrying plasmid pCgl1281, and wild-type WT-96 carrying vector pCSEK20 were spotted onto LS agar plates adjusted at indicated pH values. Growth was assessed after two days incubation at 30ºC. (B) Strains ΔCgl1281 carrying vector pCSEK20 (*circles*), ΔCgl1281 carrying plasmid pCgl1281 (*squares*), and wild-type WT-96 carrying vector pCSEK20 (*triangles*) were grown in liquid LS medium adjusted at indicated pH values at 30ºC under aerobic conditions. Growth at indicated pH values is shown relative to the growth rate (doubling /hour) at pH 7.0 in each strain. The growth rates of strains ΔCgl1281 carrying vector pCSEK20, ΔCgl1281 carrying plasmid pCgl1281, and wild-type WT-96 carrying vector pCSEK20 at pH 7.0 were 0.39, 0.44, and 0.44, respectively. Error bars indicate standard deviations from three independent experiments.

Table 1 Oligonucleotide primers

Primer	Sequence (5'→3')
pr1	AGC*GAATTC*TTTACCACCGTGGC
pr2	GTTCAGGAACCGGGTCAAA*GAATTC*
pr3	GTAAGATCTTCATCAACGTCTCGTCGT
pr4	GCGCTTTCCAGCTGAATTGTTGAGTGCAAGTGGTCGTGGCCATCGGGATTGTGCGCA
pr5	TGCGCACAATCCCGATGGCCACGACCACTTGCACTCAACAATTCAGCTGGAAAGCGC
pr6	CATAGATCTGCAAGCCAGAGCTATTG

*Bgl*II and *Eco*RI recognition sites were underlined and italicized, respectively.

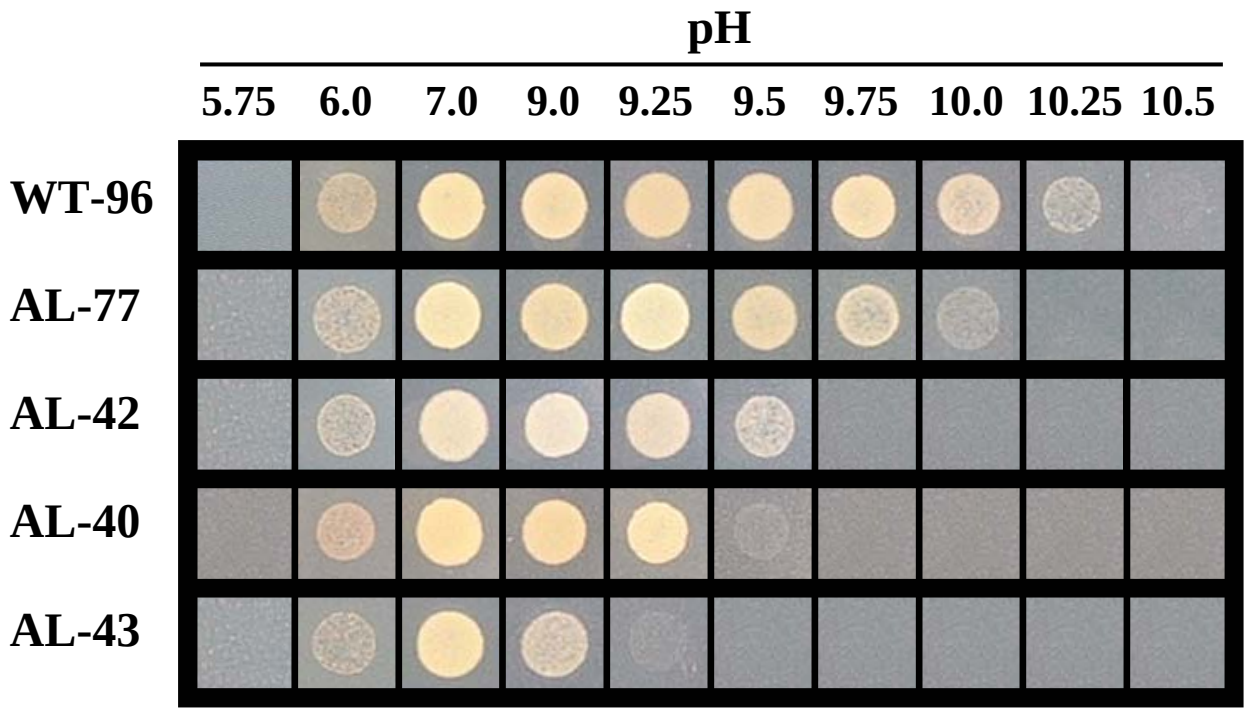

Fig. 1 ***Takeno et al.***

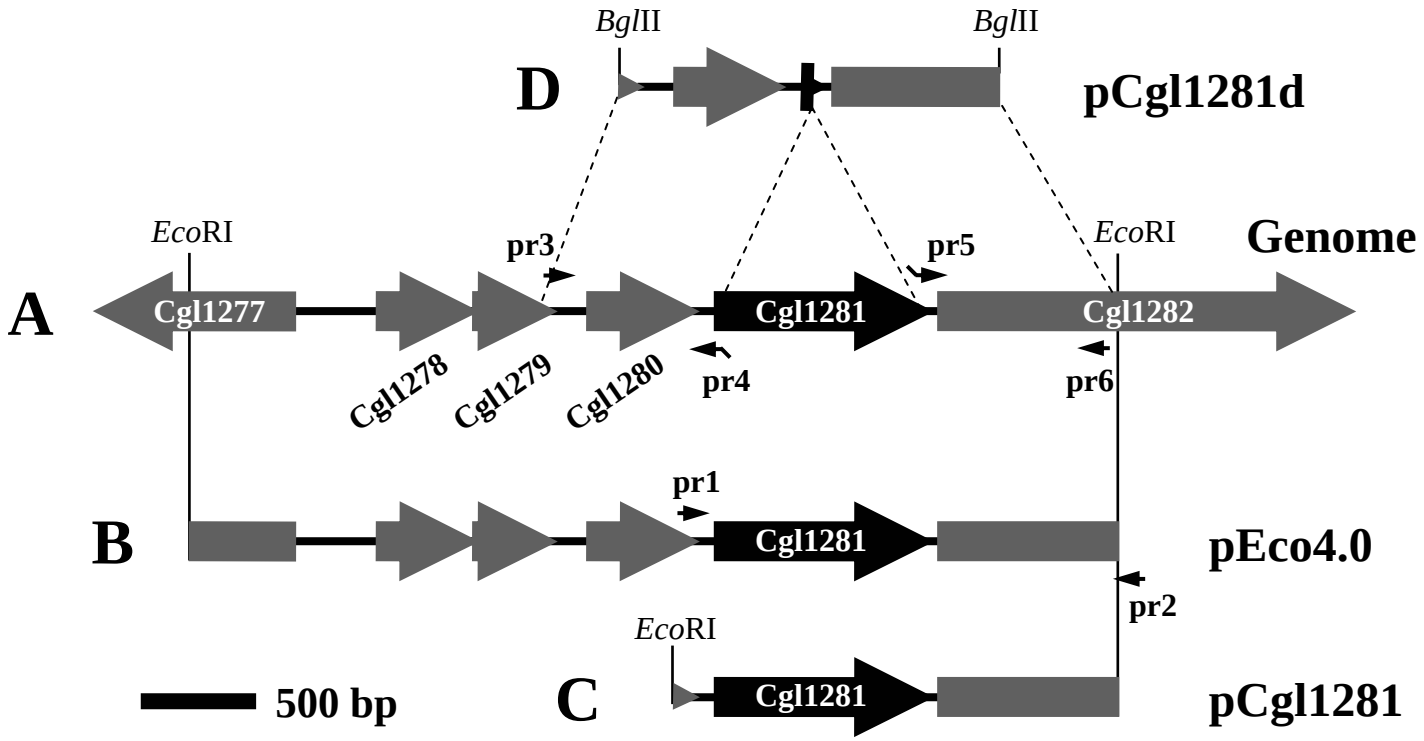

Fig. 2 *Takeno et al.*

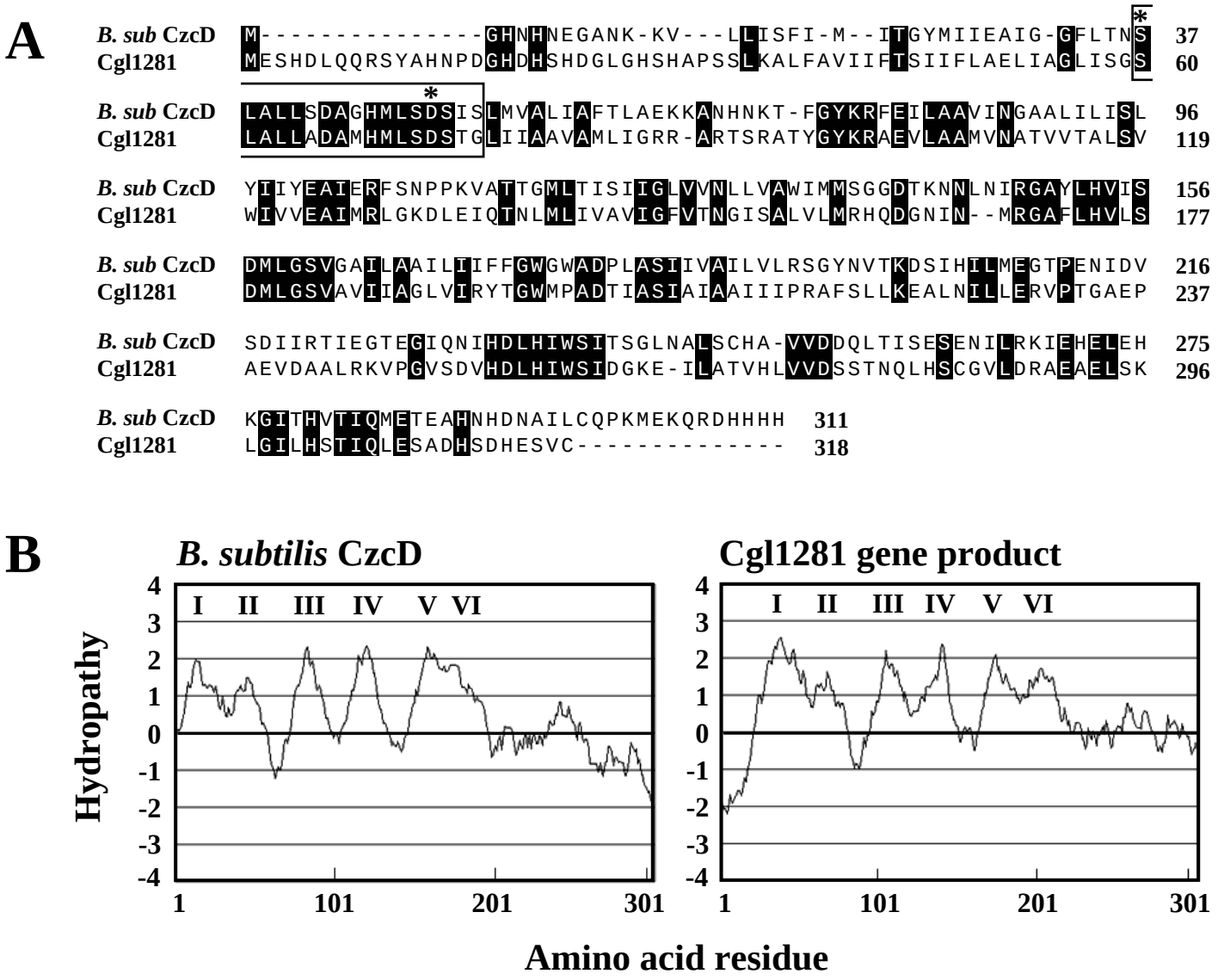

Fig. 3 ***Takeno et al.***

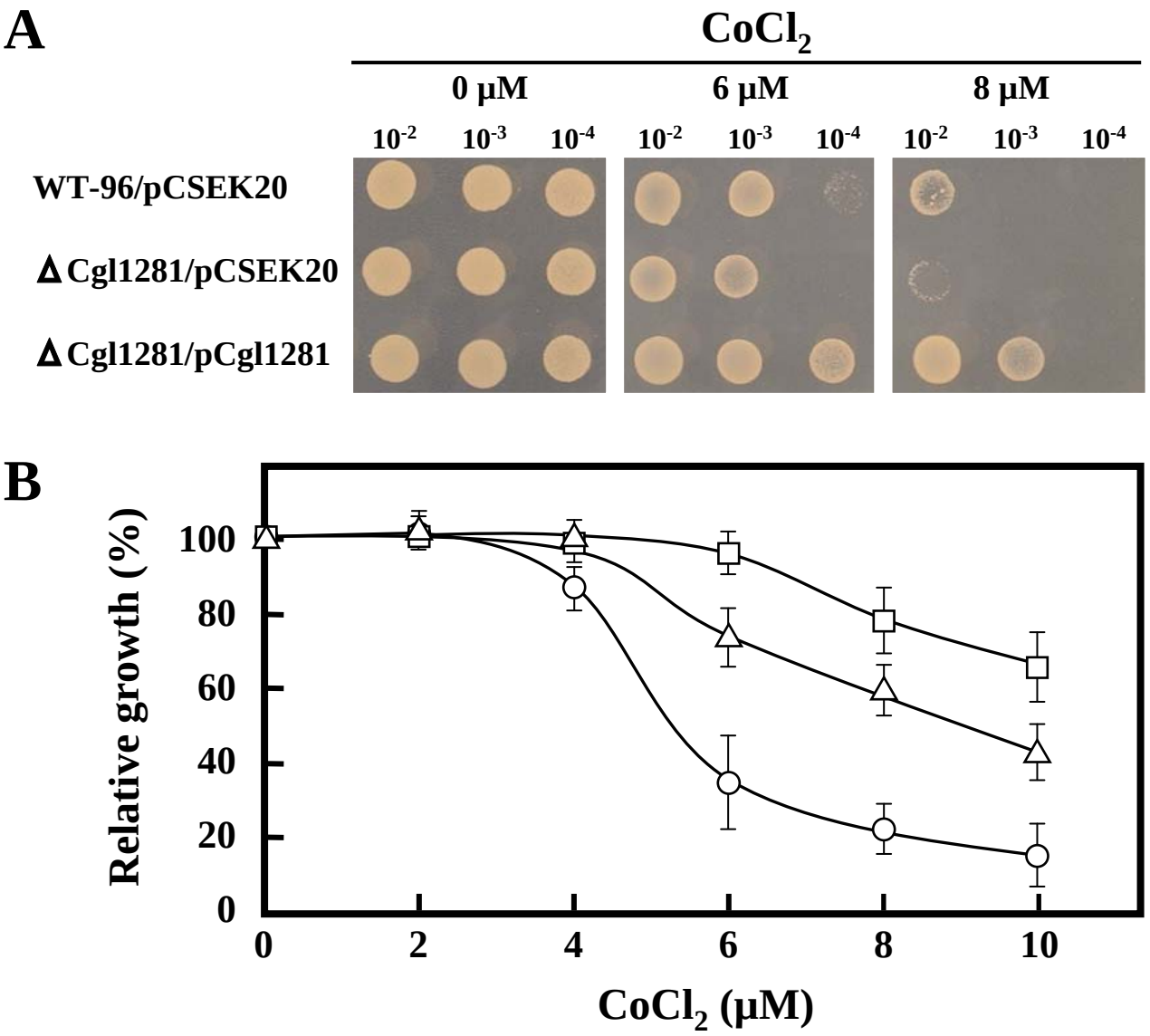

Fig. 4 *Takeno et al.*

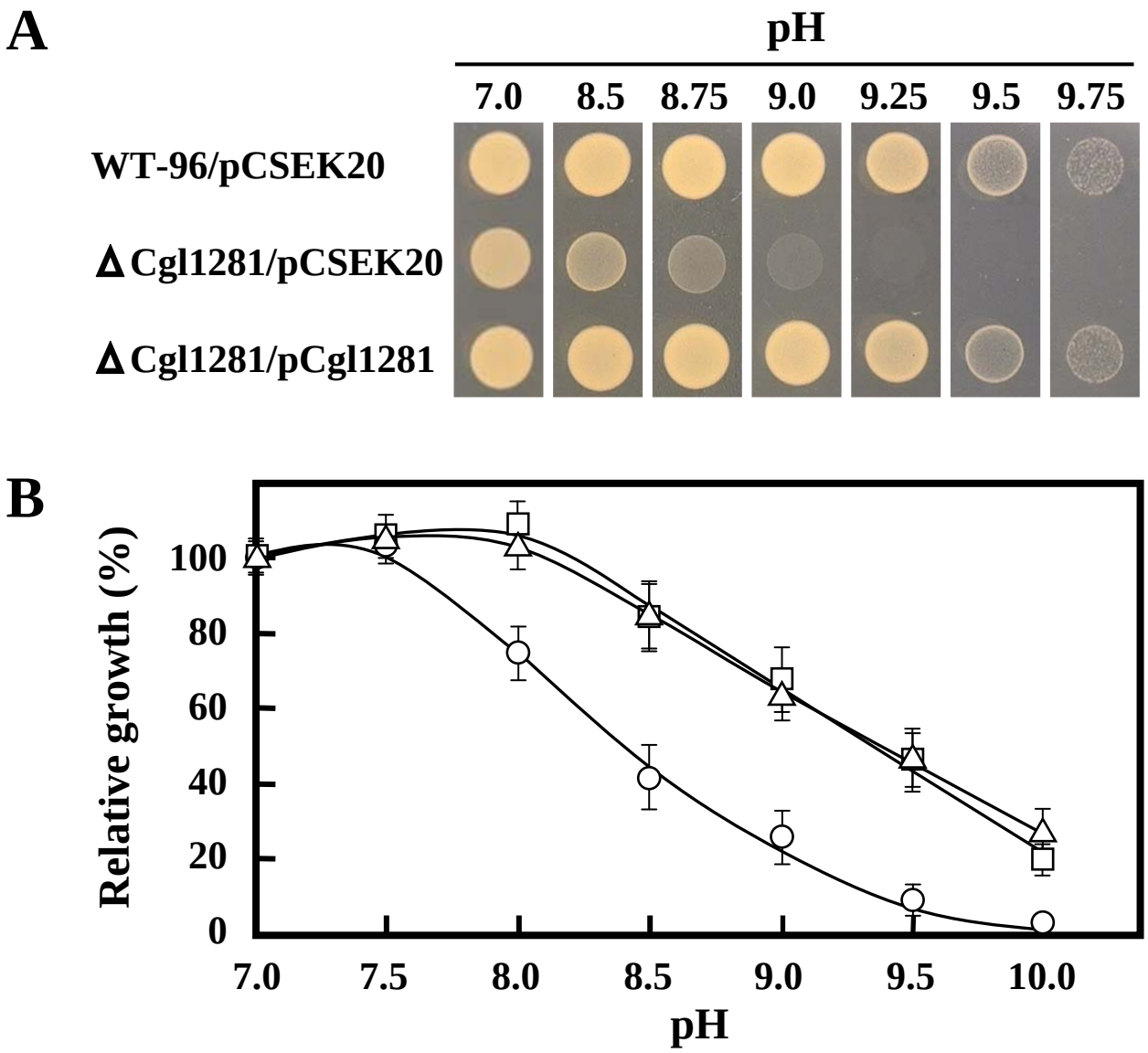

Fig. 5 ***Takeno et al.***