PssA is required for α -amylase secretion in Antarctic *Pseudoalteromonas haloplanktis*

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Extracellular protein secretion is an essential feature in bacterial physiology. The ability to efficiently secrete diverse hydrolytic enzymes represents a key nutritional strategy for all bacteria, including micro-organisms living in extreme and hostile habitats, such as cold environments. However, little is known about protein secretion mechanisms in psychrophilic bacteria. In this study, the recombinant secretion of a cold-adapted α -amylase in the Antarctic Gram-negative *Pseudoalteromonas haloplanktis* TAC125 was investigated. By a combination of several molecular techniques, the function of the *pssA* gene was related to α -amylase secretion in this psychrophilic bacterium. Deletion of the *pssA* gene completely abolished amylase secretion without affecting the extracellular targeting of other substrates mediated by canonical secretion systems. The *pssA* gene product, PssA, is a multidomain lipoprotein, predicted to be localized in the bacterial outer membrane, and displaying three TPR (tetratricopeptide repeat) domains and two LysM modules. Based on functional annotation of these domains, combined with the experimental results reported herein, we suggest a role for PssA as a molecular adaptor, in charge of recruiting other cellular components required for specific α -amylase secretion. To the best of our knowledge, no proteins exhibiting the same domain organization have previously been linked to protein secretion.

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INTRODUCTION

Protein export systems are present in all living organisms. Bacteria have evolved several complex systems for protein export. Gram-negative bacteria face a special challenge in this regard, since secreted proteins must cross the inner membrane, the periplasmic space, and the outer membrane. Secretion of proteins takes place via a variety of mechanisms, from simple one-component systems to complex multi-component pathways (Desvaux *et al.*, 2009; Johnson *et al.*, 2006).

This wide diversity in the structure of protein secretion systems can be interpreted in the light of the central role played by extracellular protein secretion in bacterial physiology. Secreted proteins provide the means by which micro-organisms interact with and modify their surrounding environment, also a crucial aspect necessary for survival

in hostile extreme habitats, such as Antarctica. Although several cold-adapted exoenzymes have been characterized, the small amount of information available on the mechanisms responsible for their extracellular addressing has come from our laboratory (Cusano *et al.*, 2006a, b; Parrilli *et al.*, 2008b, 2009; Tutino *et al.*, 2001). The development of a cold-adapted expression system (Parrilli *et al.*, 2008a) paved the way to investigate the cellular machineries devoted to protein secretion in Antarctic bacterial hosts. The α -amylase from *Pseudoalteromonas haloplanktis* TAB23 (Feller *et al.*, 1992) was chosen as a model enzyme. This enzyme is synthesized as a preproenzyme, composed of a signal peptide, the mature enzyme (49 kDa), and a C-terminal propeptide (21 kDa), which constitutes a structurally independent domain that neither exhibits any foldase function nor affects the amylase catalytic activity (Feller *et al.*, 1998). The export of the α -amylase precursor through the inner membrane likely occurs via the Sec pathway (Cao & Saier, 2003; Feller *et al.*, 1998). Once in the periplasmic space, α -amylase undergoes folding into its catalytically competent form (Feller *et al.*,

Abbreviations: T2SS, type II secretion system; TPR, tetratricopeptide repeat.

Supplementary material is available with the online version of this paper.

1998). Subsequently, secretion machinery drives its extracellular translocation. The proenzyme is found in the *P. haloplanktis* TAB23 culture supernatant as a precursor until the late exponential growth phase, when the action of an extracellular protease removes the C-terminal domain (Feller *et al.*, 1998). Although the C-terminal propeptide sequence is not homologous to that of β -autotransporters (Henderson *et al.*, 2004), in a previous paper (Feller *et al.*, 1998) it was suggested that it may function as a protein secretion helper when amylase is produced in *Escherichia coli*. However, different results were obtained when the secretion of recombinant α -amylase and its truncated version, devoid of the C-terminal domain, was investigated either in the enzyme source strain, *P. haloplanktis* TAB23, or in another Antarctic bacterium, *P. haloplanktis* TAC125 (Tutino *et al.*, 2002). It was demonstrated that the C-terminal propeptide is not mandatory for α -amylase secretion and that the presence of the C-terminal domain does not interfere either with the secretion kinetics or with the maximal secretion and production yield (Tutino *et al.*, 2002).

In the light of the above results, we decided to study the secretion of the truncated α -amylase (hereafter, ' α -amylase' indicates the protein without C-terminal domain). This approach was chosen to simplify data analysis and interpretation, since it allows ruling out the occurrence of C-terminal-domain-mediated effects on protein secretion, if any. α -Amylase secretion was studied in *P. haloplanktis* TAC125. Several reasons prompted us to use this bacterium as recombinant host: (i) it is the first Antarctic micro-organism whose genome has been sequenced and annotated (Médigue *et al.*, 2005); (ii) in this bacterium, recombinant α -amylase secretion occurs in a way similar to that in the source strain (Tutino *et al.*, 2001, 2002); (iii) *P. haloplanktis* TAC125 is devoid of endogenous α -amylase activity (Tutino *et al.*, 2001); and (iv) the cold-adapted α -amylase was successfully used as secretion carrier in a cold gene-expression system for secretion of heterologous proteins in *P. haloplanktis* TAC125 (Cusano *et al.*, 2006b; Parrilli *et al.*, 2008b).

The *in silico* analysis of the *P. haloplanktis* TAC125 genome (Médigue *et al.*, 2005) demonstrated that the bacterium possesses, besides specialized machineries for secretion of type IV pili and curli components, only one other secretion system: a type II secretion system (T2SS) (Parrilli *et al.*, 2009) homologous to already described T2SSs in many other Gram-negative bacteria (Johnson *et al.*, 2006). This secretion system is not involved in recombinant α -amylase secretion in *P. haloplanktis* TAC125 (Parrilli *et al.*, 2008b). Indeed, a *P. haloplanktis* TAC125 mutant strain in which the T2SS was knocked out was able to specifically secrete the cold-adapted α -amylase like the wild-type strain (Parrilli *et al.*, 2008b), thus suggesting the occurrence of an as yet uncharacterized secretion pathway.

In this paper we report the identification of a cold-adapted protein required for α -amylase secretion in *P. haloplanktis* TAC125.

METHODS

Bacterial strains and growth condition. *P. haloplanktis* TAC125 (Médigue *et al.*, 2005) was isolated from Antarctic sea water. *E. coli* DH5 α (Hanahan, 1983) was used as host for the gene cloning. *E. coli* EP1100 [F⁺ *mcrA* Δ (*mrr*-*hsdRMS*-*mcrBC*) ϕ 80*dlacZAM15* Δ *lacX74* *recA1* *endA1* *araD139* Δ (*ara*, *leu*)7697 *galU* *galK* λ^- *rpsL* *nupG*] was used as host for the cosmid library screening and for *in vitro* complementation.

P. haloplanktis TAC125 was grown in aerobic conditions at 15 °C in TYP broth (per litre: 16 g yeast extract, 16 g Bacto tryptone, 10 g Marine Mix) at pH 7.5, supplemented with ampicillin (200 μ g ml⁻¹) or chloramphenicol (50 μ g ml⁻¹) if transformed. *E. coli* was routinely grown in Luria–Bertani broth (Sambrook & Russell, 2001) at 20 °C or at 37 °C supplemented with ampicillin (100 μ g ml⁻¹), chloramphenicol (50 μ g ml⁻¹) or kanamycin (50 μ g ml⁻¹) if transformed. Transformation of *P. haloplanktis* was achieved by intergeneric conjugation (Parrilli *et al.*, 2008a).

Construction and screening of cosmid library. Construction of a cosmid library of *P. haloplanktis* TAC125 genomic DNA was performed as described in the pWEB cosmid cloning kit EPICENTRE brochure. The number of cosmid clones required to ensure that any given *P. haloplanktis* TAC125 DNA sequence is contained within the library is 420; the calculated total number of independent clones contained within the constructed *PhTAC125* cosmid library was 616. The cosmid clones were transformed by electroporation with pBBRamy Δ Ct and plated on selective LB plates containing 100 μ g ampicillin ml⁻¹, 50 μ g chloramphenicol ml⁻¹ and 1% soluble starch. The plates were incubated at 20 °C and colonies able to secrete amylase were detected by the amylase activity test (Filloux *et al.*, 1985). The selected clones were grown in liquid culture at 20 °C in LB medium with 100 μ g ampicillin ml⁻¹ and 50 μ g chloramphenicol ml⁻¹. The amylase activity was tested on the culture medium of each clone. The amylase secretion yield of clones displaying higher amylase activity was evaluated by assaying amylase and β -lactamase activity in the extracellular medium and corresponding intracellular extracts.

General techniques for plasmid construction. Standard methods were employed for DNA manipulation and isolation, amplification by PCR, and DNA sequencing (Sambrook & Russell, 2001; Ausubel *et al.*, 1994).

Construction of pBBRamy Δ Ct vector. To produce the *P. haloplanktis* TAB23 α -amylase lacking its C-terminal propeptide in *E. coli* EP1100 cells an expression vector, pBBRamy Δ Ct, was synthesized. The DNA fragment containing the *amy* Δ Ct gene under control of the *lacZ* promoter was excised by *TfiI/XbaI* double digestion of p α H12wt* (Feller *et al.*, 1998) followed by enzymic reaction to fill protruding ends. The DNA fragment was inserted into *AccI*-digested pBBR122 (Antoine & Locht, 1992) after fill-in of protruding ends.

Construction of cosmid subclones. *Subclone 1.* 12–26 cosmid DNA was digested with *Bgl*III and derived DNA fragments were loaded in a 0.7% agarose gel and separated by electrophoresis. The 9201 bp DNA fragment was purified from the gel and cloned into pUC18 vector (Norrandar *et al.*, 1983) previously digested with *Bgl*III.

Subclone 2. To obtain a subclone containing the cosmid region between the PSHAb0127 and PSHAb0133 genes, 12–26 cosmid DNA was digested with *Bst*II107I and the protruding ends obtained were subjected to intramolecular ligation.

Subclone 3. 12–26 cosmid DNA was digested with *Ava*I and the DNA fragments obtained were loaded in a 0.7% agarose gel and separated by electrophoresis. The 9268 bp DNA fragment was purified from the gel and cloned into pGEM vector previously digested with *Ava*I.

Subclone 4. PSHAb0139 and PSHAb0140 were amplified from *P. haloplanktis* TAC125 genomic DNA in two separate PCRs. Primers used to amplify the first fragment of 1102 bp were designed to introduce a *SphI* restriction site and to cover the natural *PvuII* restriction site (oligo 140PvuII forward and oligo 140SphI reverse; see Supplementary Table S1, available with the online version of this paper). Primers used to amplify the second fragment of 1646 bp were designed to introduce an *EcoRI* restriction site and to cover the natural *PvuII* restriction site (oligo 139EcoRI forward and oligo 140PvuII reverse; see Supplementary Table S1). The two fragments were digested with *PvuII/SphI* and *EcoRI/PvuII* respectively and ligated into pUC18 (*SphI/EcoRI*).

Subclone 5. The 12-26 cosmid DNA was digested with *BamHI* and the DNA fragments obtained were loaded in a 0.7% agarose gel and separated by electrophoresis. The 13580 bp DNA fragment was purified from the gel and cloned into pUC18 vector previously digested with *BamHI*.

Construction of pFCamy Δ Ct-pssA vector. To complement *pssA* function in *P. haloplanktis* TAC125- Δ *pssA* mutant cells the expression vector pFCamy Δ Ct-pssA was constructed. The 2504 bp region including the *pssA* gene and its upstream region was excised by *FspI* digestion of subclone 4 and then ligated into the α -amylase psychrophilic expression vector pFCamy (Cusano *et al.*, 2006b) digested by *BglII* after enzymic fill-in of protruding ends.

Amplification of the *P. haloplanktis* TAB23 *pssA* gene homologue. Two internal primers 43F and 43R (Supplementary Table S1), designed to amplify a 595 bp fragment of the *pssA* gene, were used to perform a PCR amplification on the *P. haloplanktis* TAB23 genome. The sequence of the amplified fragment was determined and aligned in the BLAST nucleotide database.

Protein electrophoresis and immunoblotting. Protein samples were analysed by SDS-PAGE (12%, w/v, acrylamide) according to standard methods (Ausubel *et al.*, 1994). For immunoblotting, the proteins were transferred to a PVDF membrane (Millipore). For immunodetection of proteins, *P. haloplanktis* TAB23 anti- α -amylase (Feller *et al.*, 1998) or anti- β -lactamase antisera were diluted in blocking buffer (phosphate-buffered saline, 5% skimmed milk). Peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) was used as secondary antibody. Proteins were detected by chemiluminescence (Pierce).

Zymographic assay. *P. haloplanktis* TAC125 wild-type and *P. haloplanktis* TAC125- Δ *pssA* mutant strains were grown at 15 °C in standard conditions and the zymographic assay was performed as previously reported (Parrilli *et al.*, 2008b).

Enzymic assays. α -Amylase activity was assayed by using the Boehringer-Roche kit AMYL under the conditions previously reported (Feller *et al.*, 1998). β -Lactamase activity was assayed according to O'Callaghan *et al.* (1972).

RESULTS

Isolation of a cosmid clone containing the *P. haloplanktis* TAC125 genomic fragment responsible for the recombinant α -amylase secretion

To identify the genes encoding the secretion machinery responsible for the extracellular addressing of the cold-

adapted α -amylase in *P. haloplanktis* TAC125, an *in vivo* heterologous complementation assay was set up.

Since *E. coli* cannot secrete the cold-adapted α -amylase devoid of its C-terminal propeptide (Feller *et al.*, 1998), this bacterium was used as a host for screening the *P. haloplanktis* TAC125 genomic DNA cosmid library, looking for cold-adapted gene product(s) allowing secretion of the α -amylase in the mesophilic host. The use of a cosmid library was appropriate because secretion pathway genes are usually organized in large gene clusters.

The cosmid library of randomly generated *P. haloplanktis* TAC125 genomic DNA fragments (~40 kb long) was obtained using the pWEB cosmid cloning kit and the recombinant cosmids were transferred into recipient cells already containing an expression vector for production of the α -amylase (pBBRamyl Δ Ct). The pBBRamyl Δ Ct plasmid was synthesized by cloning the *amyl* Δ Ct gene in the pBBR122 vector. This broad-host-range vector was chosen since its replication is compatible with the replication of the pWEB vector, while it carries two selection genes, which confer resistance to chloramphenicol and kanamycin, respectively. *E. coli* cells co-transformed with pBBRamyl Δ Ct and pWEB cosmid library clones were plated at 20 °C on a selective solid medium containing starch. In these conditions, an amylolytic halo will only surround colonies that can secrete an amylase activity in the extracellular medium.

About 600 clones were screened on plates, evaluating the size of the colonies versus the haloes, and 60 clones displaying wider amylolytic haloes were grown in liquid culture at 20 °C. Amylase activity was tested in the culture medium of each clone. Nine clones displaying higher amylase activity were subjected to further analysis. The amylase and β -lactamase activities were assayed in the extracellular medium and corresponding intracellular extracts of the nine clones (data not shown) to determine the amylase secretion yield (expressed as the percentage of total enzymic activity detected in the extracellular medium). The localization of the periplasmic β -lactamase was monitored to rule out that the extracellular targeting of the recombinant cold-adapted α -amylase was due to host outer-membrane leakage. One clone (*E. coli* clone 12-26) passed this last screening step, being able to secrete the α -amylase into the culture medium with a secretion yield of about 35%, under conditions where we did not detect significant amounts of β -lactamase (Table 1). Therefore, the production of the heterologous cold-adapted proteins encoded by the 12-26 cosmid insert did not alter the stability of the *E. coli* cell envelope, but conferred amylase secretion ability to the *E. coli* cells (Table 1).

The sequencing of the *P. haloplanktis* TAC125 genomic DNA insert contained in the 12-26 cosmid locates this fragment as the region between bp positions 152464 and 189746 of *P. haloplanktis* TAC125 chromosome b, containing the CDSs from PSHAb0127 and PSHAb0152 (see Supplementary Fig. S1A, Supplementary Table S2, and

Table 1. Extracellular α -amylase and β -lactamase activities in *E. coli* EPI100/pBBRamy Δ Ct cells transformed with the 12-26 cosmid (*E. coli*/pBBRamy Δ Ct + 12-26) at different growth phases

Enzyme activities are expressed as percentages of the total activity recovered in cells and culture supernatants. As control, the same values referring to *E. coli* cells co-transformed with pBBRamy Δ Ct and a non-secreting cosmid clone (13-64 cosmid) are reported. Each experiment was carried out in triplicate; means $\pm$ SD are shown.

Growth phase	<i>E. coli</i> /pBBRamy Δ Ct + 12-26		<i>E. coli</i> /pBBRamy Δ Ct + 13-64		<i>E. coli</i> /pBBRamy Δ Ct + pUC18	
	α -Amy	β -Lac	α -Amy	β -Lac	α -Amy	β -Lac
Early	39 % (1.4 $\pm$ 0.1 IU)	5 % (15.8 $\pm$ 4 IU)	9 % (0.28 $\pm$ 0.1 IU)	4 % (22.4 $\pm$ 5 IU)	9 % (0.33 $\pm$ 0.3 IU)	5 % (294 $\pm$ 8 IU)
Exponential	25 % (3.3 $\pm$ 0.1 IU)	16 % (62.7 $\pm$ 5 IU)	8 % (0.33 $\pm$ 0.1 IU)	5 % (20.6 $\pm$ 2 IU)	12 % (0.69 $\pm$ 0.1 IU)	10 % (448 $\pm$ 5 IU)
Stationary	34 % (7.3 $\pm$ 0.4 IU)	15 % (48.3 $\pm$ 3 IU)	7 % (0.71 $\pm$ 0.3 IU)	5 % (19.1 $\pm$ 3 IU)	13 % (1.52 $\pm$ 0.3 IU)	15 % (862 $\pm$ 4 IU)

<http://www.genoscope.cns.fr/agc/mage/psychroscope> and <http://bioinfo.hku.hk/PsychoList/>).

The selected DNA portion does not code either for a T2SS component or for any proteins of the already characterized secretion pathways. It encodes a few proteins of known function and several proteins of unknown function (Supplementary Table S2).

Construction of 12-26 cosmid subclones and test for their ability to promote α -amylase secretion

To find out which of the genes were involved in α -amylase secretion, several subclones of the 12-26 cosmid insert were constructed. Different portions of the *P. haloplanktis* TAC125 genomic DNA region between CDS PSHAb0127 and PSHAb0152 were cloned using different strategies. These subclones are illustrated in Supplementary Fig. S1B.

Subclone 1 includes a 9201 bp fragment that contains a portion of CDS PSHAb0127, full PSHAb0128 and PSHAb0129 genes, and a large section of CDS PSHAb0130. The 9268 bp DNA fragment cloned in subclone 2 includes the region between CDS PSHAb129 and CDS PSHAb134 in addition to a portion of CDS PSHAb135. In subclone 3, the region between the PSHAb0127 and PSHAb0133 genes is present. Subclone 4 contains a genomic DNA fragment that includes CDS PSHAb0139 and CDS PSHAb0140. In subclone 5, a 13 580 bp DNA fragment includes the region between PSHAb0141 and PSHAb0152. Attempts to clone the DNA region comprising PSHAb0135 to PSHAb0138 failed, although several cloning strategies were applied.

E. coli/pBBRamy Δ Ct cells were transformed with each subclone, and recombinant cells were grown in liquid culture at 20 °C. Amylase activity was tested in the culture medium of each clone. The α -amylase was detected only in the extracellular medium of *E. coli* cells transformed with subclone 4. Extracellular medium and corresponding cellular extract of *E. coli*/pBBRamy Δ Ct + subclone 4

recombinant cells were analysed by Western blotting using anti-PhTAB23 α -amylase serum (Fig. 1a) and anti- β -lactamase serum (Fig. 1b). The α -amylase was present in both extracellular and intracellular extract of *E. coli*/pBBRamy Δ Ct + subclone 4 cells, whereas the periplasmic β -lactamase remained confined to the intracellular extract of recombinant *E. coli* cells. These results indicate that subclone 4 confers to *E. coli* cells the ability to secrete the α -amylase.

Subclone 4 includes a DNA fragment that contains two CDSs, PSHAb0139 and PSHAb0140 (hereafter called *pssA*) (Supplementary Fig. S1B). *In silico* analysis revealed that PSHAb0139 codes for a putative transcriptional regulator, and this gene displays 28.27 % identity with the homologous *E. coli* gene *yfgA* (Supplementary Table S2). The protein encoded by CDS PSHAb0140 (hereafter called *PssA*) is a 577 aa lipoprotein and the sequence analysis of its leader peptide (LipoP prediction: <http://www.cbs.dtu.dk/services/LipoP>; Juncker *et al.*, 2003) predicted that it is transported to and inserted into the inner face of the outer membrane via the Lol lipoprotein sorting pathway (Tokuda & Matsuyama, 2004). Moreover, *in silico* analysis of the *PssA* amino acid sequence with the InterPro software (<http://www.ebi.ac.uk/InterProScan>; Zdobnov & Apweiler, 2001) predicted the presence of three tetratricopeptide repeat (TPR) domains (IPR001440), encompassing amino acid positions 40 and 160 of the protein, and of two LysM peptidoglycan-binding domains (IPR002482) at the C-terminal side. No significant similarities were found between the central domain of the protein and proteins present in international protein databases.

PssA protein, labelled as putative lytic cell-wall-binding lipoprotein (Supplementary Table S2), has no homologue in the *E. coli* proteome. As shown in Supplementary Fig. S1C, the gene cluster surrounding *pssA* is conserved in beta- and gamma-proteobacteria. Furthermore, in the genomes of many Gram-negative bacteria the CDSs ranging between PSHAb0134 and PSHAb0142 are in synteny (filled rectangles in Supplementary Fig. S1C), with the sole exception of *pssA*.

Fig. 1. Analysis of α -amylase localization in *E. coli*/pBBRAmy Δ Ct cells transformed with subclone 4. Samples from *E. coli*/pBBRAmy Δ Ct transformed with pUC18 or 12-26 cosmid were used as negative and positive controls respectively. (a) Western blot analysis with anti-*P. haloplanktis* α -amylase polyclonal antiserum of extracellular samples (S) and corresponding cellular extract (P). (b) Western blot analysis with anti-*E. coli* β -lactamase polyclonal antiserum of extracellular supernatant (S) and corresponding cellular extract (P).

Identification of *pssA* gene orthologues in *P. haloplanktis* TAB23 and other Gram-negative bacteria

A PCR experiment (data not shown) carried out on *P. haloplanktis* TAB23 genomic DNA using internal primers designed on the *pssA* sequence led to the specific amplification of a 595 bp DNA fragment. The sequence of the amplified fragment showed 99% identity with the *pssA* sequence, indicating the presence of a PssA-coding gene homologue in the α -amylase source strain.

The PssA sequence was used as template for a homology search in protein databases, and gene products having a similar domain organization to PssA were found in three marine bacteria only: *Colwellia psychrerythraea* (Méthé *et al.*, 2005), *Pseudoalteromonas tunicata* D2 (GenBank AAOH000000000) and an unclassified *Alteromonadales* strain named TW-7 (GenBank AAVS000000000).

Construction and phenotype analysis of the *P. haloplanktis* TAC125- Δ *pssA* mutant

To assess the role of PssA protein in α -amylase secretion, a *P. haloplanktis* TAC125 mutant was constructed in which the *pssA* gene was knocked out.

Functional inactivation of the *P. haloplanktis* TAC125 *pssA* gene was achieved by two-step gene deletion mutagenesis. The mutation was obtained by using a suicide vector (pVS), suitably constructed for *P. haloplanktis* TAC125 (Parrilli *et al.*, 2006, 2008b). The Δ *pssA* mutated gene is characterized by a 607 bp deletion that results in a sequence frame shift.

Total RNA was extracted from the *P. haloplanktis* TAC125- Δ *pssA* deletion mutant and subjected to reverse transcriptase RT-PCR analysis to evaluate transcription of the *pssA* and PSHAb0139 genes. The deletion mutagenesis carried out in *P. haloplanktis* TAC125- Δ *pssA* abolished *pssA* gene transcription while it did not affect PSHAb0139 gene expression (data not shown). The growth behaviour of *P. haloplanktis* TAC125- Δ *pssA* in standard conditions

remained comparable to that of the wild-type strain (data not shown), suggesting that inactivation of the *pssA* gene does not affect cell viability.

To evaluate whether inactivation of the *pssA* gene may alter the general secretion ability of the bacterium in a non-specific way, for instance by interfering with a process that could indirectly impair all protein secretion mechanisms, the secretion of extracellular proteases by *P. haloplanktis* TAC125- Δ *pssA* was evaluated. This group of extracellular enzymes was analysed since it was previously demonstrated (Parrilli *et al.*, 2008b, 2009) that the majority of extracellular proteases in *P. haloplanktis* TAC125 are secreted by the type II secretory pathway. Extracellular protease secretion in *P. haloplanktis* TAC125- Δ *pssA* was investigated by gelatin zymography of culture supernatant. As shown in Fig. 2, the extracellular medium of the *P. haloplanktis* TAC125- Δ *pssA* mutant contains a number of proteolytic activities similar to those detected in the wild-type.

PssA is necessary for α -amylase secretion in *P. haloplanktis* TAC125

Secretion of cold-adapted α -amylase by *P. haloplanktis* TAC125- Δ *pssA* was investigated. The mutant strain was transformed with pFCamy Δ Ct, a vector previously constructed for the recombinant secretion of α -amylase in *P. haloplanktis* (Cusano *et al.*, 2006b). The recombinant mutant strain was grown at 15 °C and the α -amylase secretion was evaluated by Western blot analysis of intracellular and extracellular protein samples withdrawn at different growth phases. As shown in Fig. 3(a), the α -amylase was present only in the intracellular extracts, demonstrating that the inactivation of the *pssA* gene completely abolishes the secretion of this enzyme in *P. haloplanktis* TAC125- Δ *pssA*.

To exclude the possibility that the inability of *P. haloplanktis* TAC125- Δ *pssA* cells to secrete the recombinant α -amylase was due to a polar effect on expression of *pssA* neighbouring genes, a complementation experiment

Fig. 2. Protease zymography of *P. haloplanktis* TAC125 wild-type (wt) and *P. haloplanktis* TAC125- $\Delta pssA$ mutant ($\Delta pssA$) culture supernatants. Samples were withdrawn at the mid-exponential phase and concentrated tenfold. The zymographic developing time was 18 h, a condition that ensures the detection of all proteases contained in the sample.

was carried out. The complementing plasmid, pFCamy Δ Ct-*pssA*, was constructed to restore PssA function in *P. haloplanktis* TAC125- $\Delta pssA$ cells. This plasmid contains both the *amy* Δ Ct gene and the DNA sequence encoding *pssA* and its upstream region (150 bp long), in which the presence of a putative promoter sequence was predicted (SoftBerry BPROM software: <http://linux1.softberry.com/berry.phtml>). The mutant strain was transformed with pFCamy Δ Ct-*pssA* and the recombinant cells were grown in standard conditions. Intracellular and extracellular protein samples were withdrawn at different growth phases, and subjected to Western blotting analysis with anti-*P. haloplanktis* α -amylase (Fig. 3b) and anti-*E. coli* β -lactamase sera (Fig. 3c). As shown in Fig. 3(b, c), the α -amylase was detected in the extracellular samples, while the β -lactamase was always fully associated with the intracellular extracts, thus demonstrating that the secretion of the α -amylase is partially restored in *P. haloplanktis* TAC125- $\Delta pssA$ /pFCamy-*pssA* complemented cells.

DISCUSSION

Over the last several years our group has been involved in unravelling the molecular mechanism involved in the secretion of the cold-adapted α -amylase from the marine Antarctic bacterium *P. haloplanktis* TAB23. This α -amylase is still one of the most cold-tolerant proteins intensively studied to clarify the structure/function relationships which underlie enzyme cold-adaptation (D'Amico *et al.*, 2006). This cold-adapted enzyme is likely translocated by a Sec system in the periplasmic space, where it attains its catalytically competent folded state (Feller *et al.*, 1998), and

then an as yet unidentified secretion machinery catalyses its extracellular translocation.

The development of genetic tools for manipulation of Antarctica bacteria (Parrilli *et al.*, 2008a) allowed us to demonstrate that the C-terminal propeptide is not mandatory for α -amylase recombinant secretion either in the source strain or in the related strain *P. haloplanktis* TAC125 (Tutino *et al.*, 2002). Indeed, the propeptide can be replaced by other protein domains without affecting the secretion of the chimeric proteins (Cusano *et al.*, 2006b; Parrilli *et al.*, 2008b). Therefore it can be assumed that the mature α -amylase (i.e. devoid of the C-terminal propeptide) contains some molecular signals responsible for its specific secretion. To investigate the nature and structure of these secretion signals, mature α -amylase was subjected to a deletion mutagenesis analysis (Cusano *et al.*, 2006a). Features necessary for exoenzyme secretion were localized in a α -amylase domain, and a likely 3D recognition between α -amylase and the cognate cold-adapted secretion machinery was suggested (Cusano *et al.*, 2006a).

These results may point towards the involvement of a T2SS secretion pathway (Johnson *et al.*, 2006), which is the only canonical secretion system identified *in silico* in the *P. haloplanktis* TAC125 genome. However our recent results demonstrated that the extracellular targeting of the cold-adapted α -amylase does not depend on the T2SS in *P. haloplanktis* TAC125 (Parrilli *et al.*, 2008b).

To identify cellular components involved in the secretion of α -amylase, a *P. haloplanktis* TAC125 genomic DNA cosmid library was constructed and screened in *E. coli*. By this *in vivo* complementation experiment a *P. haloplanktis* TAC125 genomic fragment was selected, which did not encode any of the components of previously characterized secretion pathways. Data reported in this paper allowed us to identify a protein (PssA) required for the secretion of α -amylase in *P. haloplanktis* TAC125. The presence of a PssA homologue was demonstrated only in *P. haloplanktis* TAB23, i.e. the α -amylase source strain, and in three marine bacteria.

In silico analysis suggests that PssA is a lipoprotein that may be anchored into the inner face of the outer membrane. The PssA protein is predicted to contain three TPR domains; the TPR is an imperfect 34 aa repeat that is found as a protein-protein interaction module that mediates the assembly of multiprotein complexes (Blatch & Lässle, 1999; D'Andrea & Regan, 2003; Das *et al.*, 1998).

Recently, two lipoproteins equipped with TPR domains have been related to the functionality of the bacterial type IV pilus biogenesis system, which closely resembles the T2SS (Peabody *et al.*, 2003), and reported data (Carbannelle *et al.*, 2005; Nudleman *et al.*, 2005) indicate that TPR-containing lipoproteins have the potential to interact with and/or form an outer-membrane multiprotein complex.

The PssA protein contains also two LysM domains in its C-terminal region. This protein module was originally

Fig. 3. Western blot analysis of extracellular samples (S) and corresponding cellular extracts (P) collected at different growth phases of *P. haloplanktis* TAC125- Δ pssA/pFCamy Δ Ct (a), *P. haloplanktis* TAC125- Δ pssA/pFCamy Δ Ct-pssA (b, c) and *P. haloplanktis* TAC125/pFCamy Δ Ct (positive controls; d, e). In (a), (b) and (d), Western blot analysis was carried out by using anti-*P. haloplanktis* α -amylase polyclonal antiserum, while in (c) and (e) anti-*E. coli* β -lactamase polyclonal antiserum was used.

identified in enzymes that degrade bacterial cell walls but it is also present in many other bacterial proteins (Birkeland, 1994). The available data suggest that the LysM domain is a general peptidoglycan-binding module (Bateman & Bycroft, 2000).

In summary, PssA is a putative multidomain lipoprotein predicted to be localized in the inner leaflet of the outer membrane and displaying three TPR domains and two LysM modules. To the best of our knowledge, no proteins exhibiting the same domain organization have previously been related to protein secretion systems. Based on the functional annotation of TPR and LysM domains, combined with experimental evidence reported in the present work, a model for the role of PssA protein is suggested (Fig. 4) in which PssA interacts with other proteins and/or with itself while bound to the peptidoglycan, allowing the specific secretion of cold-adapted α -

amylase. According to the proposed model, the lack of PssA completely abolishes α -amylase secretion. Symmetrically, PssA overproduction may alter the secretion complex stoichiometry, affecting amylase secretion efficiency. A similar effect was reported when some proteins of a multicomponent secretion system were overproduced (Ball *et al.*, 1999). This may account for the partial recovery of amylase secretion in the complemented strain (Fig. 3b), where *pssA* was supplied in multiple copies.

Surprisingly, the production of the PssA protein permits recombinant *E. coli*/pBBRAmy Δ Ct+subclone 4 cells to secrete the cold-adapted α -amylase. The most plausible explanation of this result is that PssA can recruit some *E. coli* proteins, forming a protein complex able to recognize and secrete the α -amylase. Data presented in this paper do not allow speculation on structural similarities and/or

Fig. 4. Model for the role of PssA protein. The protein is proposed to work as a molecular adaptor, able to interact with other proteins (outer-membrane and/or periplasmic ones) while bound to the peptidoglycan, allowing the specific secretion of the cold-adapted α -amylase. OM, outer membrane; IM, inner membrane.

differences between the protein complexes responsible for amylase secretion in *P. haloplanktis* TAC125 and recombinant *E. coli*/pBBRam Δ Ct + subclone 4 cells. This aspect is currently under investigation and will be the subject of a following paper.

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