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Poster Presentation

Cell engineering of *Pseudoalteromonas haloplanktis* TAC125: construction of a mutant strain with reduced exo-proteolytic activity

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Background

We have already shown that using cold-adapted bacteria as host vectors, some "intractable" proteins can be efficiently produced at temperature as low as 4°C [1,2]. Furthermore, we set up a "cold" gene-expression system implemented for the secretion of recombinant proteins in the Antarctic Gram-negative bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125). Such a system could effectively conjugate the positive effect of low temperature on the recombinant product solubility with the obvious advantages linked to extra-cellular protein targeting. This novel system makes use of the psychrophilic α-amylase from *Ph*TAB23 [3] as secretion carrier. Several chimerical proteins were produced and used to test the versatility and efficiency of the novel secretion system. All the chimerical proteins were efficiently produced and secreted (Cusano AM, Ph. D thesis 2005 Università di Napoli "Federico II"). However, bacteria belonging to *Pseudoalteromonas* genus are reported to secrete a wide range of exo-proteins, especially proteases. This feature could hamper both applicability and efficiency of the cold-adapted secretion system, due to the possible recombinant product degradation.

The *Ph*TAC125 genome sequence [4] was recently determined. The *in silico* genome analysis highlighted the presence of a putative Type II secretion system (T2SS), while the extra-cellular targeting of the cold α-amylase depends on a still uncharacterized secretion pathway [4].

Results

Construction of the Ph*TAC125 suicide vector*

Figure 1 shows the Vs suicide vector constructed to generate *Ph*TAC125 genomic mutants. It is characterised by the presence of: i) the pJB3-derived *oriT* (1), a DNA fragment

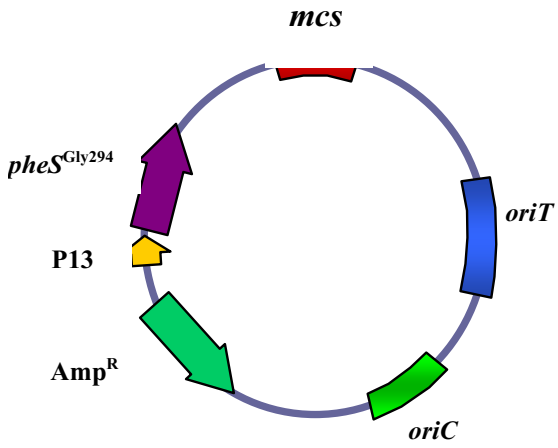

Figure 1
Schematic representation of the *Ph*TAC125 Vs suicide vector. mcs, Multiple cloning site; *oriT*, origin of conjugative transfer [1]; *oriC* and Amp^R, origin of replication and beta-lactamase encoding gene from the pUC18 vector; P13, synthetic psychrophilic promoter; $pheS^{Gly294}$, *E. coli* gene encoding a mutated version of the α subunit of Phe-tRNA synthase.

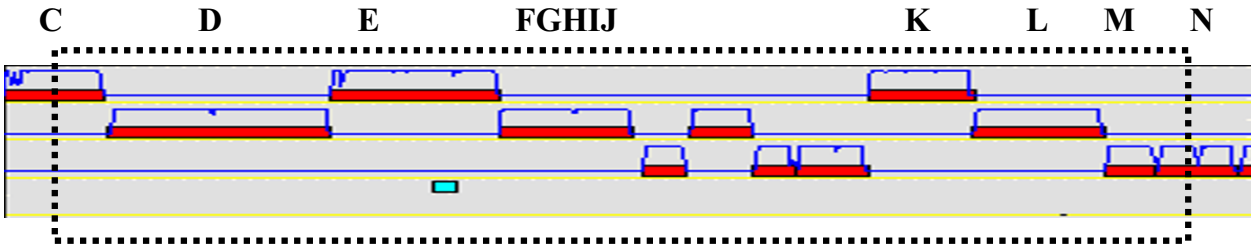

Figure 2
Genetic organization of the *Ph*TAC125 *gsp* cluster and *gspCN* deletion. The *Ph*TAC125 *gsp*$^-$ mutant was generated by deleting a genomic region corresponding to that displayed into the dotted rectangle.

responsible for the initiation of the conjugative transfer between an *Escherichia coli λpir* strain (donor) and the psychrophilic cells (acceptor); ii) the *E.coli blaM* gene, encoding a mesophilic β-lactamase which is used as selection gene to isolate the first site-specific integration event; iii) *pheS*^{Gly294}, which encodes a mutated version of the *E. coli* α subunit of Phe-tRNA synthase [5], which renders bacteria sensitive to *p*-chlorophenylalanine. This phenylalanine analog is used as counterselective agent for the isolation of those strains in which a second recombination event occurred. To assure a proper level of *pheS*^{Gly294} expression, its transcription was subjected to the control of a psychrophilic synthetic promoter (P13).

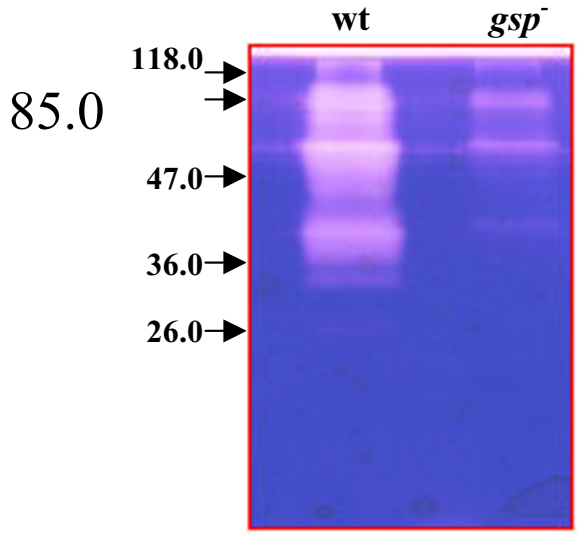

Figure 3
Gelatin zymography of *Ph*TAC125 wt and *gsp*$^-$ supernatants. Psychrophilic cells were grown in TYP medium at 4°C till late exponential phase. Culture supernatants were recovered by culture centrifugation, 10 times concentrated and loaded onto a 10% SDS-PAGE containing bovine gelatin. After the electrophoresis run, the gel was washed to remove the Na-SDS and incubated in the development solution overnight at 15°C. Finally the gel is stained with Comassie blue and destained. Molecular weight markers were marked in kDa.

Construction of a Ph*TAC125* gsp$^-$ *[Δ*gspCN*] strain*

To inactivate the T2SS pathway in *Ph*TAC125 (Figure 2), a deletion strategy was applied. Two genomic fragments were PCR amplified by using specific oligonucleotides as primers. They correspond to the 5' 360 bp portion of *gspC* and 3' 300 bp portion of *gspN* respectively. The fragments were suitably digested and cloned into the Vs vector. The resulting vector (VsCN) was mobilized by intergeneric conjugation [1] into *Ph*TAC125, and the cells were plated at 4°C on TYP solid medium containing 30 μg/ml carbenicillin to select those in which a single recombination event occurred. Second recombination event was induced by repeated plating of mutant psychrophilic cells at 4°C on minimum solid medium containing 20 mM *p*-ClPhe. The occurrence of the correct deletion was checked by sequencing the specific PCR fragments.

Phenotypic characterization of Ph*TAC125* gsp$^-$ *strain*

The global exo-proteolytic activity of the *Ph*TAC125 *gsp*$^-$ strain was analyzed by *in gel* zymography and compared to that of the wild type strain. As shown in figure 3, culture supernatant of *gsp*$^-$ strain contains a reduced number of exo-proteases.

Conclusion

We report here a cell engineering approach to the construction of a *Ph* TAC125 strain with reduced exo-protease activity. By applying a gene-placements strategy, we obtained a mutant strain in which the gene cluster encoding the T2SS was almost totally deleted. While the growth behavior and some physiological features of the *gsp*$^-$ mutant are indistinguishable from the wild type ones, the deleted strain displays a remarkable reduction in the pro-

tease content in the culture supernatant. This aspect makes the *Ph*TAC125*gsp*⁻ mutant a promising host for the recombinant secretion into the host extra-cellular medium of proteins with biotechnological potential.

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