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ORIGINAL PAPER

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Proteomic identification of a two-component regulatory system in *Pseudoalteromonas haloplanktis* TAC125

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Abstract The capability of microorganisms to utilize different carbohydrates as energy source reflects the availability of these substrates in their habitat. Investigation of the proteins involved in carbohydrate usage, in parallel with analysis of their expression, is then likely to provide information on the interaction between microorganisms and their ecosystem. We analysed the growth behaviour of the marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 in the presence and in the absence of different carbon source. A marked increase in the optical density was detected when L-malate was added to the growth medium. Bacterial proteins differently expressed in the presence of L-malate were identified by proteomic profiling experiments. On the basis of their relative increase, six proteins were selected for further analyses. Among these, the expression of a putative outer membrane porin was demonstrated to be heavily induced by L-malate. The presence of a functionally active two-component regulatory system very likely controlled by L-malate was found in the upstream region of the porin gene. A non functional genomic porin mutant was then constructed showing a direct involvement of the protein in the uptake of L-malate. To the best of our knowledge, the occurrence of such a regulatory system has never been reported in Pseudoalteromonads so far and might constitute a key step in the development of an effective inducible cold expression system.

Keywords Psychrophiles · *Pseudoalteromonas haloplanktis* TAC125 · Carbon source response · Two-component regulatory system · L-malate

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Introduction

The ability of microorganisms to utilize carbohydrates as energy source likely reflects the availability of these substrates in their habitat. Different sugars, provided to bacteria as single source of carbon and energy, require the induction of different uptake systems and metabolic enzymes to support growth and cell survival (Cases and de Lorenzo 2005). Exploration of the repertoire of carbohydrate usage-proteins, in parallel with analysis of the regulation of their expression, is then likely to reveal much about relevant metabolic features of the microorganisms providing also information about the interactions with their ecosystem.

Bacteria that live in complex environments and are exposed to frequent changes in available nutrients and to different environmental insults (soil, plants, enteric bacteria, etc.) have correspondingly complex sensor–response–control subsystems (Cases and de Lorenzo 2005). These bacteria display a large number of environmental sensors and corresponding control circuits to invoke a wide variety of contingent responses. By contrast, bacteria that live in a more constant host

environment such as obligate symbiotic bacteria, extremophiles, rickettsias, etc., generally have simple regulatory structures that involve a much lower number of genes (van Ham et al. 2003; Wilcox et al. 2003).

A combined investigation of metabolic changes and regulatory expression mechanisms following adaptation of bacteria to differences in the availability of carbon sources can be provided by proteomic investigations. Expression proteomic studies are used to measure up- and down-regulation of protein levels in the cell, thus providing differential protein expression profiles following a number of intra- and/or extracellular stimuli (Alban et al. 2003).

With the aim to investigate the molecular mechanisms of adaptation to changes in available nutrients of bacteria that live in stable niches, we analysed the growth behaviour of the marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) (Birolo et al. 2000), grown in minimal medium both in the presence and in the absence of different carbon sources. Bacterial proteins differently expressed in the presence of L-malate were identified by exploiting proteome expression profiling experiments, taking advantage by our recent achievement of the entire genome sequence of *Ph*TAC125 (Medigue et al. 2005). Among several proteins whose expression is affected by the selected carbon source, we focused our attention on the *PSHAb0363* gene coding for a putative outer membrane porin. The organization of its upstream region was investigated by transcriptional fusion experiments, strongly suggesting the occurrence of a functional two-component sensor regulatory system for C4–dicarboxylic acids (Janausch et al. 2002). Data obtained demonstrated that the expression of the porin gene was remarkably affected by the presence of L-malate in the growth medium. Finally, a non functional porin mutant was constructed and its growth behaviour in the presence of L-malate was analysed showing a direct involvement of the outer membrane porin in the transport and uptake of L-malate.

Experimental procedures

Strains and growth conditions

Pseudoalteromonas haloplanktis TAC125 (*Ph*TAC125) (Birolo et al. 2000), a member of the gamma-proteobacteria was grown in aerobic conditions at 16°C in modified DSMZ medium 79 (a minimal synthetic sea water medium containing 1 g/l KH_2PO_4, 1 g/l NH_4NO_3, 10 g/l NaCl, 0.2 g/l $MgSO_4 \times 7H_20$, 10 mg/l $FeSO_4$, 10 mg/l $CaCl_2 \times 2H_2O$), supplemented with casamino acid 0.5% w/v and 0.2% w/v carbon source. Care was taken to use in all experiments the same casamino acid batch (Difco Laboratories GmbH, Augsburg, Germany: batch 0231-01).

Genomic mutant of the outer membrane porin (*Ph*TAC125*ΔPSHAb0363::*Amp^+) was constructed by allelic replacement with an ampicillin resistance cassette (Kast 1994).

Preparation of protein extracts

Bacteria were harvested at the time points indicated, washed in 10 mM Tris, pH 7.5, 10 mM EDTA, and resuspended in the same buffer containing 1.4 μM phenyl methyl sulfonyl fluoride. Cells were disrupted by passage through a French Press. After centrifugation (20,000×*g*, 4°C, 45 min) the protein concentration of the extract was determined with the RotiNanoquant Kit (Roth, Karlsruhe, Germany).

2D PAGE

For isoelectric focusing, protein extracts (300 μg proteins) were loaded onto commercially available IPG-strips (pH 4–7; Amersham Biosciences) according to Bernhardt et al. (Bernhardt et al. 1999). In the second dimension polyacrylamide gels of 12.5% acrylamide and 2.6% bisacrylamide were used. Gels were stained with Colloidal Coomassie Brilliant Blue. For the Coomassie staining, a 5% w/v stock solution of Coomassie Brilliant Blue G-250 was prepared. This stock solution was then used to make up the staining solution containing 10% w/v $(NH_4)_2SO_4$, 1% v/v phosphoric acid, and 2% v/v (final concentrations) of the Coomassie stock solution. After fixation in a 40% ethanol/10% acetic acid v/v solution, the gels were stained for about 20 h in the staining solution diluted with ethanol (20% v/v, final concentration). Excess Coomassie stain was removed by washing the gels in distilled water.

Protein identification

Proteins were identified by MS. All the protein spots detectable by Coomassie stain were excised from gels with the Proteome Works Spot Cutter System (Bio-Rad). In-gel trypsin digestion of the proteins and extraction of the peptides were mostly done in the Ettan Spot Handling Workstation (Amersham Biosciences). Peptide masses were measured either in a Voyager-DE STR or in a Proteomics Analyzer 4700 (both Applied Biosystems). Peptide mass fingerprints were analysed using an in-house database developed from the genome sequence of *Ph*TAC125. The resulting MS data were analysed with the Bioanalyst Software (Applied Biosystems) and the integrated Mascot (Matrix Science) script.

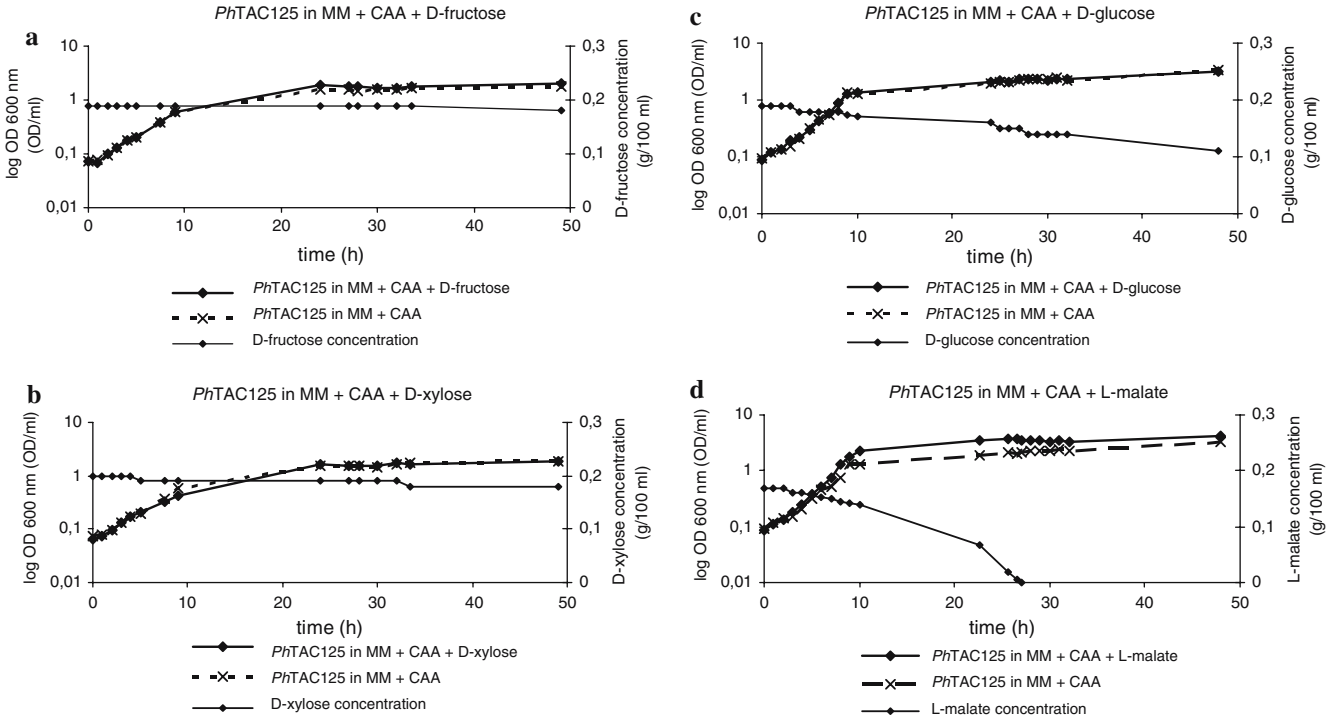

Fig. 1 Growth profiles of *Ph*TAC125 grown at 16°C in minimal medium (*MM*) supplemented with casamino acids (*CAA*) in the presence and in the absence of different carbon sources and the consumption of the substrate. **a** D-fructose. **b** D-xylose. **c** D-glucose. **d** L-malate

Denomination of the proteins

Proteins are numbered according to the genome annotation (Medigue et al. 2005).

Transcriptional fusion assays

Reporter assays of transcriptional fusions were performed by measuring β-galactosidase activity as described by Duilio et al. 2004.

Results and discussion

Carbon source response of *Ph* TAC125

The growth behaviour of the marine Antarctic bacterium, *Pseudoalteromonas haloplanktis* TAC125 (*Ph*TAC125) in response to different carbon sources was investigated. Bacteria were grown at 16°C in minimal marine salt medium supplemented with a complex amino acids mixture (casamino acids) in the presence and in the absence of D-glucose, D-fructose,

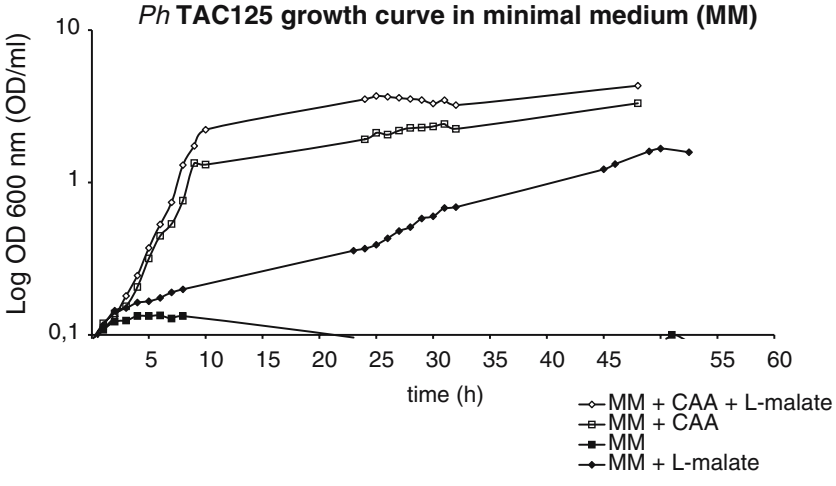

Fig. 2 Growth behaviour of *Ph*TAC125 at 16°C in minimal medium (*MM*) in the presence and in the absence of either casamino acids (*CAA*) or L-malate or both as carbon and energy source

D-xylose and L-malate, respectively. Figure 1 shows the corresponding growth profiles together with the consumption of the individual carbon sources. These results demonstrated that *Ph*TAC125 is unable to degrade D-fructose and D-xylose, the concentration of both carbon sources in the medium being virtually unchanged after 50 h. D-glucose was very slowly metabolized by *Ph*TAC125; after 50 h only 40% of this substrate was actually used. In contrast, *Ph*TAC125 showed a marked increase in the optical density when grown in the presence of L-malate together with a corresponding consumption of the carbon source (Fig. 1).

The growth behaviour of *Ph*TAC125 in the presence of L-malate was then further investigated in details. Bacterial cells were grown at 16°C in minimal medium supplemented with casamino acids both in the presence and in the absence of 0.2% L-malate. Control experiments were performed by growing *Ph*TAC125 cells either in minimal medium only or in minimal medium

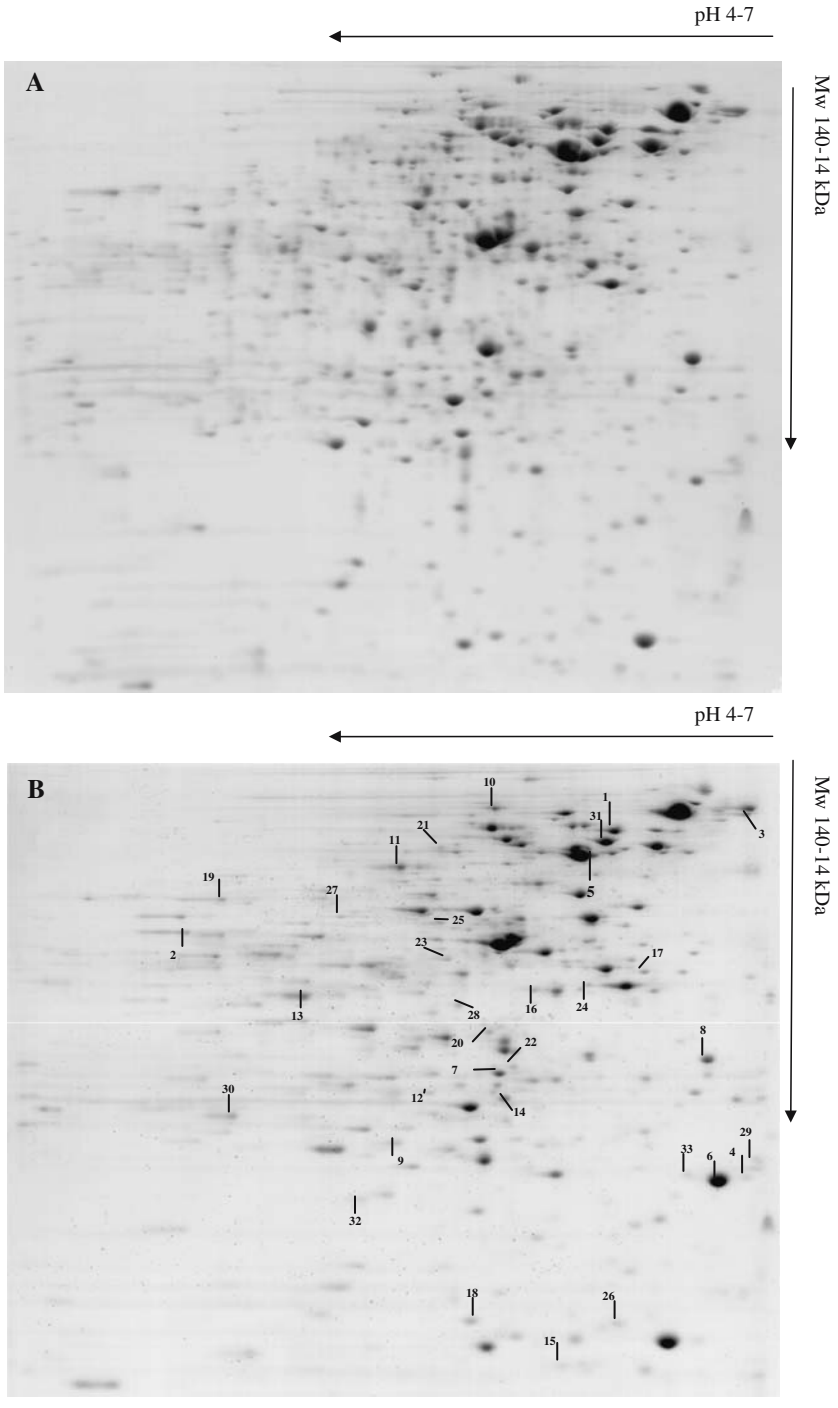

Fig. 3 2D-gel electrophoretic analysis of the intracellular protein fraction of *Ph*TAC125 during growth in minimal medium supplemented with casamino acids in the absence (**a**) and in the presence (**b**) of L-malate. A pH 4–7 IPG gradient was chosen for isoelectric focusing. Protein spots were detected by colloidal Coomassie blue staining and are numbered according to Table 1

Table 1 *Pseudoalteromonas haloplanktis* proteins differently expressed from cells grown in the presence of L-malate

Spot	Function	pI	MW (kDa)	Accession number
Cell envelope and membrane-associated cellular processes				
1	Putative enzyme with a phosphatase-like domain	6.52	10.2	PSHAa2478
2	Na(+)-translocating NADH-quinone reductase α subunit	5.52	48.6	PSHAa2241
3	Putative TonB-dependent receptor	4.38	92	PSHAa1840
4	Putative outer membrane porin	4.55	34.4	PSHAa2567
5	Iron-regulated outer membrane virulence protein homolog	4.81	73.3	PSHAb0251
6	Outer membrane protein W (OmpW) family	4.98	23.5	PSHAa2155
7	Putative outer membrane porin	4.98	38.4	PSHAb0363
8	Putative flagellin (FliC-like)	4.64	40.2	PSHAa0782
9	Ubiquinol-cytochrome c reductase, iron-sulphur subunit	7.7	20.9	PSHAa2530
Intermediary metabolism				
10	Bifunctional protein [Includes aconitate hydratase]	4.96	93.9	PSHAa0184
11	Succinate dehydrogenase flavoprotein subunit	5.84	64.6	PSHAa1649
12	Succinyl-CoA transferase, subunit A	5.44	25.1	PSHAa1447
13	Citrate synthase	5.74	48.1	PSHAa1653
14	Transaldolase B	5.24	35	PSHAa2559
15	Putative fructose-bisphosphate aldolase	5.56	33.1	PSHAb0548
16	Probable isopropylmalate dehydrogenase	6.14	35.7	PSHAb0465
17	4-hydroxyphenylpyruvate dioxygenase	4.64	38.8	PSHAa2168
18	Nucleoside diphosphate kinase	5.5	15.5	PSHAb0142
19	IMP dehydrogeanse	8.12	52	PSHAa0648
20	Alpha keto acid dehydrogenase complex, beta subunit	5.13	35.4	PSHAa1631
21	Alpha keto acid dehydrogenase complex, E2 component	5.32	57.3	PSHAa1630
22	Malonyl-CoA-[acyl-carrier-protein] transacylase	4.95	32.3	PSHAa1809
23	3-oxoacyl-[acyl-carrier-protein] synthase I	5.2	42.6	PSHAa2080
Information transfer pathways				
24	Nucleoid-associated protein	5.46	34.5	PSHAa2381
25	Adenylosuccinate synthetase	5.38	47.6	PSHAa0275
26	DNA-directed RNA polymerase omega chain	4.87	10.2	PSHAa2791
27	Putative ATP-dependent RNA helicase	9.32	48	PSHAb0411
28	Phenylalanine tRNA synthetase, alpha-subunit	5.38	37.3	PSHAa1904
29	50S ribosomal subunit protein L4	9.76	21.9	PSHAa0145
30	30S ribosomal subunit protein S4	10.12	23.4	PSHAa2807
31	Chaperone protein DnaK	4.59	68.7	PSHAb0357
Unknown proteins				
32	Conserved hypothetical protein	8.88	27.8	PSHAa0394
33	Putative membrane-associated protein with TPR-like domain	4.87	22.2	PSHAb0136

Proteins were clustered into functional groups according to the genome annotation (Medigue et al. 2005)

supplemented with L-malate. Results are shown in Fig. 2. *Ph*TAC125 cells are unable to grow in minimal medium without an added carbon source, as expected. However L-malate alone was able to support cellular growth when added to the medium. The enhancing effect of L-malate on the growth of *Ph*TAC125 was clearly observed also when bacterial cells were grown in the presence of casamino acids. Interestingly, the growth rate of exponentially growing cells was only marginally affected by L-malate whereas a marked increase in the length of exponential phase was observed when L-malate was present, with an increase in the total optical density higher than 50%.

Proteomic profiles of *Ph* TAC125 cells

The protein profile of *Ph*TAC125 grown in minimal medium supplemented with casamino acids was constructed using two-dimensional gel (2-D) electrophoresis. The concentration of casamino acids was carefully selected to support a cellular growth able to produce the

Table 2 List of the six most abundant proteins expressed from *Pseudoalteromonas haloplanktis* cells in the presence of L-malate

Spot	Protein	pI	MW (KDa)	Accession number
6	Outer membrane protein W (OmpW) family	4.98	23.5	PSHAa2155
7	Putative outer membrane porin	4.98	38.4	PSHAb0363
8	Putative flagellin (FliC-like)	4.64	40.2	PSHAa0782
10	Bifunctional protein [aconitate hydratase]	4.96	93.9	PSHAa0184
13	Citrate synthase	5.74	48.1	PSHAa1653
14	Transaldolase B	5.24	35	PSHAa2559

Table 3 Plasmids and oligonucleotides

Plasmid	Description	References
pPLB	Promoter-trap cold-adapted vector containing the promoter-less *Ph*TAE79 *lacZ* gene	Duilio et al. (2004)
P(PSHAa2155)	pPLB containing the PSHAa2155 promoter region (231 bp)	This work
P(PSHAa0782)	pPLB containing the PSHAa0782 promoter region (307 bp)	This work
P(PSHAa1653)	pPLB containing the PSHAa1653 promoter region (407 bp)	This work
P(PSHAa0184)	pPLB containing the PSHAa0184 promoter region (234 bp)	This work
P(PSHAa2559)	pPLB containing the PSHAa2559 promoter region (232 bp)	This work
P(PSHAb0363)	pPLB containing the PSHAb0363 promoter region (3437 bp)	This work
Oligonucleotide		
PSHAa2155 Fw	5′ GCTTTAGAAATCTAGACTGAAGAAACAGC	This work
PSHAa2155 Rev	5′ GTTCTCCAAGATATCTTTGTGATTGCC	This work
PSHAa1653 Fw	5′ GAGATAGTCTAGATTATTTGAAGCACAACG	This work
PSHAa1653 Rev	5′ CCATCGATATCTCTCCTATTAAGATAAC	This work
PSHAa0782 Fw	5′ GAGATAGTCTAGATTATTTGAAGCACAAC	This work
PSHAa0782 Rev	5′ GCCATGATATCACTCCTGATTACTTTCG	This work
PSHAa0184 Fw	5′ CACTAAAGCAGCTCTAGAAGAAAACCG	This work
PSHAa0184 Rev	5′ GTAGCACAAGATATCCCTCTTGGTGACG	This work
PSHAa2559 Fw	5′ CAGGTGATCTAGAAGTGATCATAGTTAGC	This work
PSHAa2559 Rev	5′ CGTTAGATATCTGCTTTCCTCATTCC	This work
PSHAb0363A Fw	5′ CCAAAGCTAGGATCCGCTTAATTATAC	This work
PSHAb0363A Rev	5′ CCTGGATCCAATATCGATAGTTTTACG	This work
PSHAb0363B Fw	5′ GATGGACGTCTAGAACTATCGATATTAG	This work
PSHAb0363B Rev	5′ CCTTCAATCTAGATATCTGCAGGAGTATC	This work
PSHAb0363C Fw	5′ GATACTTCCTGCAGATATATTAATTG	This work
PSHAb0363C Rev	5′ CCTGTGTCCCGGGTATCATCATGTGTCC	This work

appropriate amount of protein extract needed for further proteomic analysis. A pH 4-7 IPG gradient was chosen for isoelectric focusing followed by a second dimension on SDS-PAGE. Figure 3a shows the resulting electrophoretic gel. All protein spots stained by Colloidal Coomassie blue G-250, were excised from the gel and digested with trypsin. Proteins were identified by Matrix-Assisted Laser Desorption Ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of the corresponding peptide digests. Accurate peptide mass values were then used to search for a complete protein databank of *Ph*TAC125 taking advantage of the recently determined genome sequence of the bacterium (Medigue et al. 2005).

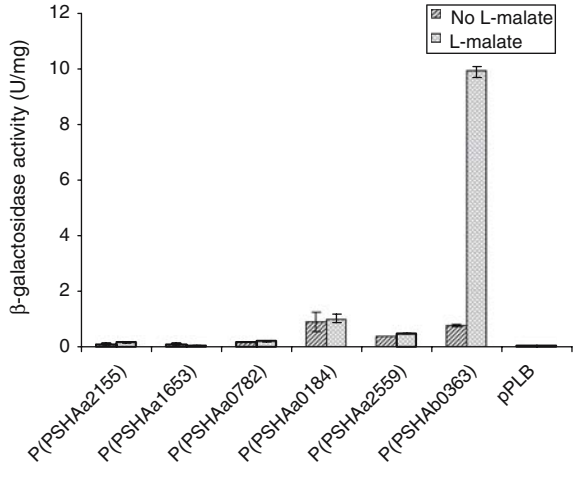

Fig. 4 Profiles of the *β*-galactosidase activity of the *P*(PSHA*X*)*::-lacZ* transcriptional fusion constructs measured in *Ph*TAC125 cells grown in minimal medium supplemented with casamino acids in the presence and in the absence of L-malate. pPLB, negative control

Investigation of protein expression changes in *Ph*TAC125 cells caused by the presence of L-malate in the growth medium was carried out by using the same approach. Bacterial cells were grown as described above in the presence of 0.2% L-malate. Protein extracts were collected in the late exponential phase when the maximum consumption of the carbon source was observed. The protein mixture was fractionated by 2-D gel electrophoresis as described above and the resulting protein profile was compared to that obtained in the absence of L-malate. A total of 33 proteins differently expressed in the presence of L-malate was identified (Fig. 3b; Table 1). Approximately 30% of them were proteins involved in membrane associated processes such as transport and binding of proteins and lipoproteins or chemotaxis and motility events. Approximately 45% of all proteins were metabolic enzymes but only 16% of them are directly involved in the metabolism of carbohydrates and related molecules.

Among all the proteins identified, six proteins were selected for further transcriptional analysis as their expression was particularly increased in the presence of L-malate.

Transcriptional analysis of the genes coding for the differently expressed proteins of *Ph*TAC125

Table 2 shows the list of the six proteins that, considering their relative abundance in the gel, were chosen for

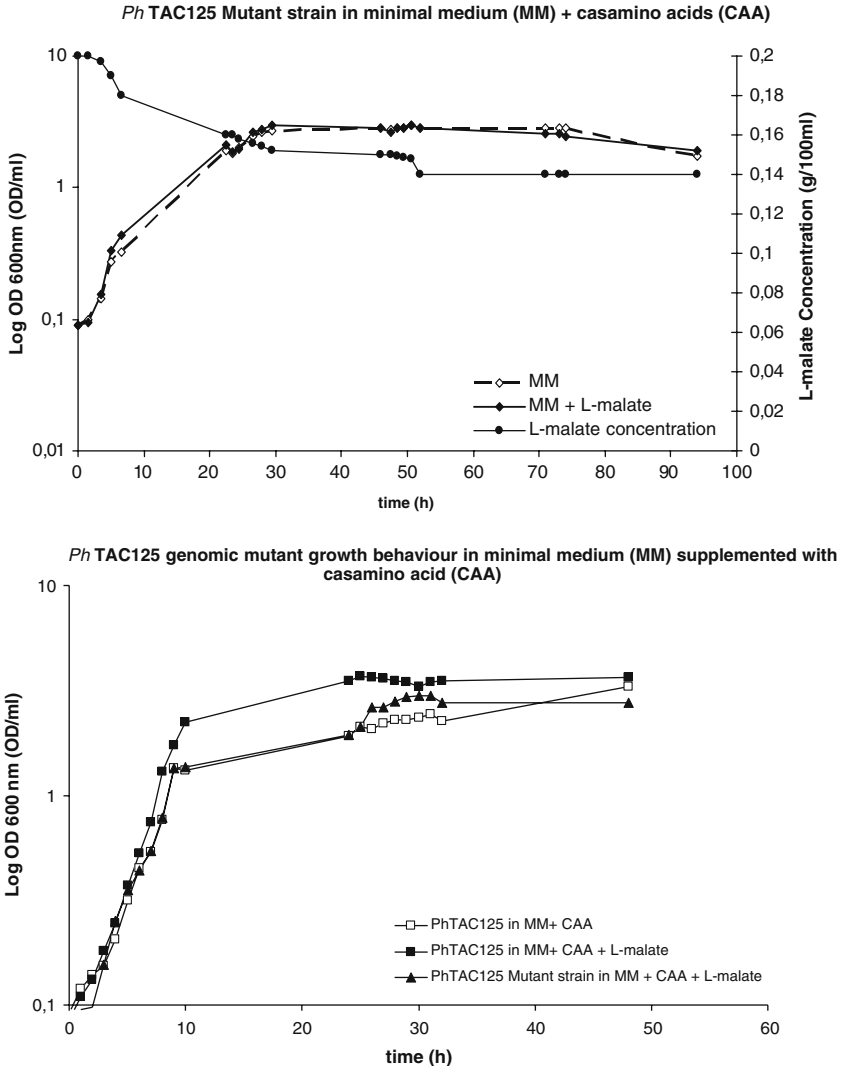

Fig. 5 Growth profiles of *Ph*TAC125 porin mutant strain grown at 16°C in minimal medium (*MM*) supplemented with casamino acids (*CAA*) in the presence and in the absence of L-malate and the consumption of the substrate (**a**). Growth profile of *Ph*TAC125 porin mutant strain grown at 16°C in minimal medium (*MM*) supplemented with casamino acids (*CAA*) in the presence of L-malate in comparison with growth profiles of *Ph*TAC125 wild type strain grown at 16°C in minimal medium (*MM*) supplemented with casamino acids (*CAA*) in the presence and in the absence of L-malate (**b**)

the transcriptional analysis. Three of these are key enzymes of the carbohydrates metabolism and the remaining are involved in protein transport/binding and motility processes. According to the literature, the genes coding for these proteins are all transcriptionally regulated (Cai and Winkler 1997; Cunningham et al. 1997; Davies et al. 1999; Hayashi et al. 2002; Kromer et al. 2004; Lai et al. 2004; Shen et al. 2004; Thompson et al. 2002; Tang et al. 2004); therefore their differential expression might be ascribed to the activation of transcriptional stimuli induced by the presence of L-malate.

The regulation of the six genes was investigated by transcriptional fusion experiments. The DNA regions located upstream the six genes were individually fused to a promoter-less *lacZ* gene contained in pPLB plasmid (Duilio et al. 2004), generating the P(PSHA*X*) vectors (Table 3).

Recombinant *Ph*TAC125 cells harbouring the β-galactosidase gene individually fused to the six promoters were grown in minimal medium either in the absence or in the presence of 0.2% L-malate. Figure 4 shows the β-galactosidase activity measured at the mid-stationary phase. All recombinant *Ph*TAC125 cells transformed with the constructs displayed notably higher β-galactosidase activity when compared to the control vector (pPLB). However, in most cases, the β-galactosidase activity remained unchanged following L-malate supply. In contrast, *Ph*TAC125 cells harbouring the putative outer membrane porin promoter region showed a 13-fold activity increase when L-malate was added to the medium.

The transcriptional activity of the six genomic regions was further investigated by monitoring the β-galactosidase activity during the exponential and the late-stationary growth phase. These experiments demonstrated that the transcriptional activity of the six promoters was not growth-phase dependent (data not shown).

As a whole, these results indicated that five of these genes were not directly affected by L-malate; however the amount of the corresponding protein products were found largely increased in *Ph*TAC125 grown in the presence of L-malate. This finding suggests that the

promoters of these genes are not directly controlled by L-malate but a more complex and indirect regulation mechanism might be responsible for their higher expression.

On the contrary, data shown in Fig. 4 demonstrated that the regulatory element located upstream of the porin gene is directly and remarkably influenced by the presence of L-malate in the growth medium. This DNA region has a rather specific organization. Two CDSs respectively encoding a putative C4-dicarboxylates sensor kinase and a putative C4 response regulator are located in this region. Furthermore, a CDS coding for a periplasmic transporter of dicarboxylic acids was found downstream of the porin gene. This association strongly suggests the occurrence of a two-component sensor regulatory system (Stock et al. 2000), that is affected by the presence of L-malate.

However, since the DNA region used in the fusion was quite large, the unambiguous identity of the element induced by L-malate, whether the two-component system or the porin promoter itself, could not be established.

Construction of a non functional porin mutant

With the aim to investigate the involvement of the outer membrane porin in the transport and uptake of L-malate in *Ph*TAC125 cells, a non functional mutant was constructed and characterized.

The *Ph*TAC125 porin mutant strain was grown at 16°C in minimal medium supplemented with casamino acids both in the presence and in the absence of 0.2% L-malate. Figure 5a shows that the growth profiles of porin mutant cells in the two conditions were nearly identical. Accordingly, a very slow consumption of the substrate by *Ph*TAC125 porin mutant was observed, with only 30% of L-malate used after 90 h.

The growth behaviour of *Ph*TAC125 porin mutant was then compared to that observed for the wild type strain grown in the presence and in the absence of 0.2% L-malate. The results shown in Fig. 5b demonstrated that the growth profile of the porin mutant strain was almost identical to that observed for *Ph*TAC125 wild type cells grown without L-malate. Accordingly, the increase in the length of exponential phase observed in wild type *Ph*TAC125, was not detected in the porin mutant strain when L-malate was added.

These results strongly suggest that the outer membrane porin is directly involved in the transport and uptake of L-malate in *Ph*TAC125.

Conclusions

The overall results presented in this paper suggest that *Ph*TAC125 contains a functional two-component regulatory system very likely controlled by L-malate. This element regulates the expression of a specific outer membrane porin involved in the uptake of L-malate. To the best of our knowledge, the occurrence of such a regulatory system has never been reported in Pseudoalteromonads so far. Finally, the identification of a regulatory element induced by L-malate might constitute a key step in the development of an effective inducible cold expression system.

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