Proteomic analysis of a non-virulent mutant of the phytopathogenic bacterium *Erwinia chrysanthemi* deficient in osmoregulated periplasmic glucans: change in protein expression is not restricted to the envelope, but affects general metabolism

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Osmoregulated periplasmic glucans (OPGs) are general constituents of the envelope of Gram-negative bacteria. They are required for full virulence of bacterial phytopathogens such as *Pseudomonas syringae*, *Xanthomonas campestris* and *Erwinia chrysanthemi*. *E. chrysanthemi* is a pectinolytic γ -proteobacterium that causes soft rot disease on a wide range of plant species. In addition to the loss of virulence, *opg* mutants exhibit a pleiotropic phenotype that affects motility, bile-salt resistance, exoenzyme secretion, exopolysaccharide synthesis and membrane lipid composition. This is believed to be the first proteomic analysis of an OPG-defective mutant of *E. chrysanthemi* and it revealed that, in addition to the effects described, catabolic enzyme synthesis was enhanced and there was a greater abundance of some proteins catalysing the folding and degradation of proteins needed for various stress responses. Thus, in the *opg* mutant strain, loss of virulence was the result of a combination of envelope structure changes and cellular metabolism modifications.

INTRODUCTION

Osmoregulated periplasmic glucans (OPGs) are general periplasmic constituents of the envelope of many Gram-negative bacteria. Their common features are that (i) glucose is the sole constitutive sugar, and (ii) their abundance in the periplasm increases as the osmolarity of the medium decreases (Bohin & Lacroix, 2006). OPG synthesis mutants of phytopathogenic bacteria fail to incite disease in their plant hosts (Bohin & Lacroix, 2006). Mutants of *Pseudomonas syringae* pv. *syringae* devoid of OPGs show a reduced virulence on the common bean and its plant host, and fail to elicit a hypersensitive response on tobacco, a non-host plant (Bohin & Lacroix, 2006; Loubens *et al.*, 1993). *Xanthomonas campestris* pv. *vesicatoria* deficient in OPG biosynthesis shows an attenuated virulence in tomato (Minsavage *et al.*, 2004). *Erwinia chrysanthemi* *opg* mutants are completely non-virulent when inoculated into potato tubers or chicory leaves (Page *et al.*, 2001). In *E. chrysanthemi*, synthesis of the glucose backbone of OPGs is catalysed by both of the products of the *opgGH* operon: OpgG and OpgH catalyse

the synthesis of linear glucans containing 5–13 glucose units joined by $\beta(1\rightarrow2)$ linkages and branched by $\beta(1\rightarrow6)$ linkages (Cogez *et al.*, 2001). Mutations in *opgG* or *opgH* completely abolish OPG synthesis (Page *et al.*, 2001).

E. chrysanthemi causes soft rot disease in a wide range of plant species, including crops of economic importance, such as vegetables and ornamentals. The virulence of *E. chrysanthemi* is strongly associated with the synthesis and secretion of exoenzymes, particularly pectinases and, to a lesser extent, cellulases and proteases. Pectinases and cellulases are secreted through a type II secretory apparatus known as Out, while proteases are secreted by a type I secretory system. Action of these enzymes causes maceration of the plant tissues, leads to the extension of the disease throughout the plant and provides bacteria with carbon sources (Hugouvieux-Cotte-Pattat *et al.*, 1996). In addition to the non-virulent phenotype, *opgG* or *opgH* mutants exhibit a pleiotropic phenotype: colonies are mucoid (exopolysaccharide overproduction), and motility, bile-salt resistance and secretion of exoenzymes are reduced (Page *et al.*, 2001). The *Opg*[−] phenotypes observed may be explained by a modification in envelope composition. One could hypothesize that the absence of virulence for *opgG* mutants is a consequence of the reduction of the secretion

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Abbreviations: OPG, osmoregulated periplasmic glucans; TCA, tricarboxylic acid.

of exoenzymes. An *outC* mutant, defective in the Out type II secretory system, is more severely affected in pectinase secretion than an *opgG* mutant. When inoculated into potato tubers or chicory leaves, the virulence of the *outC* mutant is severely reduced, but not abolished. Co-inoculation experiments with a mix of a mutant strain and the wild-type strain were performed in potato tubers. *outC* mutant cells grew normally in the mix, while no growth was observed for the *opgG* mutant cells contained in the mix. Thus, loss of virulence of *opgG* mutants does not result simply from a deficiency in exoenzyme secretion (Page *et al.*, 2001). In addition, some of the OPGs are released into the medium by wild-type cells, indicating that OPGs play a role only within the periplasmic space of *E. chrysanthemi* (Cogez *et al.*, 2001). It has often been reported that lack of an envelope component affects the surface properties of bacteria, and thus the interaction of bacteria with their environment.

In this paper, a comparative analysis of the soluble proteome of wild-type cells and *opgG* mutant cells of *E. chrysanthemi* was performed. Synthesis of several proteins governing essential cellular processes, such as protein folding and degradation, and carbohydrate catabolism, was increased. This suggests that the non-virulent phenotype of the *opgG* mutant is a complex phenomenon that includes, but is not restricted to, changes in the surface properties of the envelope.

METHODS

Bacterial strains and media. Wild-type *E. chrysanthemi* (EC3937) and its *opgG::uidA-Kan* (NFB3500) (Page *et al.*, 2001) derivative

strain, were grown at 30 °C, with vigorous shaking, in Luria–Bertani broth (LB) medium that contained 10g bactotryptone l⁻¹ and 5g yeast extract l⁻¹ (Difco), but did not contain NaCl (low-osmolarity medium, 90 mosmol l⁻¹).

Extraction of proteins. Bacteria were grown to mid-exponential phase, harvested by centrifugation and washed with 0.1 M phosphate buffer, pH 6.6, containing 20 mM MgSO₄ and a protease inhibitor cocktail (Complete; Roche). The cell pellets were resuspended in the same buffer. Cells were disrupted by passing through a precooled French pressure cell at 1.4 × 10⁷ Pa. The lysates were centrifuged at 16 000 g at 4 °C for 30 min to separate supernatants and pellets. The supernatants were treated by a mix of DNase I (10 µg ml⁻¹) and RNase A (10 µg ml⁻¹) at 4 °C for 30 min and then centrifuged at 45 000 g at 4 °C for 30 min. Four volumes of acetone were added to the supernatants and they were incubated at -20 °C for 1 h before centrifugation at 13 000 g for 15 min. The pellets were washed with 75 % ethanol, dried and resuspended in solubilization buffer [7 M urea, 2 M thiourea, 4 % (w/v) CHAPS (Sigma-Aldrich), 65 mM DTT (Proteomic Solutions), 0.25 mM Triton X-100 and a protease inhibitor cocktail]. Protein concentration was determined by the Bradford test using BSA (Protein assay standard II; Bio-Rad) as a standard (Bradford, 1976). Total protein concentration was determined according to Stickland (1951).

Electrophoresis of proteins. IEF was performed using 18 cm IPG strips (Immobiline DryStrip; GE Healthcare), with a linear gradient of pH ranging from 3 to 10. Protein solutions (600 µg in 200 µl of solubilization buffer) were mixed with 200 µl rehydration buffer [8 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 100 mM DTT, 2 % (v/v) ampholytes (pH 3–10; Proteomic Solutions) and 0.001 % bromophenol blue]. After 16 h rehydration, IEF was performed using a pHaser isoelectric focusing system (Proteomic Solutions) at 20 °C for 10 000 Volt hours (V h), from 0 to 5000 V over 24 h. Before the second dimension, strips were immersed for 20 min in equilibration buffer [6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, 0.375 M Tris/HCl (pH 8.8) and 2 % DTT]. SDS-PAGE was carried out on a 12 % w/v polyacrylamide resolving gel (Duracryl 30 %; Proteomic

Fig. 1. Silver-stained 2D gels of the soluble proteome from exponential-phase bacteria grown in LB medium without NaCl. Left, *E. chrysanthemi* EC3937; right, the *opgG*-derivative strain NFB3500.

Solutions) in running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS, pH 8.3). Proteins were visualized by staining gels with silver nitrate solution, according to Rabilloud (1999).

Analysis of 2D electrophoresis gels. Image analysis of 2D electrophoresis gels was performed using the Melanie Image Master 2D Platinum 5 software (GE Healthcare) and three gels from each condition. The proteome profile of wild-type *E. chrysanthemi* EC3937 was used as a reference pattern for spot analysis and all spot files were matched to the reference pattern. The intensity of each spot was normalized by total spot intensity.

Protein identification by MALDI-TOF MS. Spots were excised from gel and washed three times with 50 μ l 25 mM ammonium bicarbonate and once with 50 % acetonitrile in 25 mM ammonium bicarbonate. The supernatants were discarded and the gel pieces were dried. The proteins were digested in-gel with 20 ng porcine trypsin μ l⁻¹ (Promega) in 25 mM ammonium bicarbonate. The digestion was performed overnight at 37 °C. The resulting peptides were extracted from the gel by 1 % formic acid and then by 50 % acetonitrile in 1 % formic acid. Supernatants from both the mutant and the wild-type were pooled and peptides were dried and

resuspended in 0.5 % formic acid. A 0.5 μ l volume of the peptide solution was directly mixed with 1 μ l 2,5-dihydroxybenzoic acid matrix [10 mg ml⁻¹ in methanol/water (7:3, v/v)] on the stainless steel target and allowed to crystallize in a gentle stream of air. Peptide mass fingerprint spectra were manually acquired on a MALDI-TOF MS instrument (DE STR Pro; Applied Biosystems). An in-house MASCOT (Matrix Sciences; <http://www.matrixscience.com/>) search engine was used for protein identification against the *E. chrysanthemi* database (<http://asap.ahabs.wisc.edu/asap/ASAP1.htm>).

RESULTS AND DISCUSSION

Growth of the *E. chrysanthemi* *opgG* mutant

Bacterial cells were grown in LB medium without NaCl, and growth was found to be similar for wild-type cells (EC3937) and *opgG* mutant cells (NFB3500). Total protein concentration was measured using a 50 ml culture sample taken at mid-exponential phase (OD₆₂₀ 0.4), and only a slight difference, if any, was observed between the two strains: 0.12

Fig. 2. Enlargement of the silver-stained 2D gel of the soluble proteome of the *E. chrysanthemi* *opgG*-derivative NFB3500. The numbers indicate spots that were identified. Spots A, B, C and D are landmarks for Melanie Image Master 2D Platinum 5 software calibration.

Table 1. Identification of proteins differentially expressed in the *opgG* mutant cells of *E. chrysanthemi*

Spot no.* and function	Accession no.†	pI	Mass (kDa)‡	Gene†	Location	Ratio‡	Putative function†	Coverage	Score§	No. of peptides matched
Polysaccharide synthesis										
25	15220	5.4	51.2	<i>cpsG</i>	Cytoplasm	2.3	Phosphomannomutase	22	67	10
18	15224	6.2	35	<i>fcl</i>	Cytoplasm	1.6	GDP-L-fucose synthetase	27	52	7
33	20562	5.9	36.9	<i>galE</i>	Cytoplasm	2.8	UDP-glucose 4-epimerase	25	68	7
32	19489	5.8	32.3	<i>galF</i>	Cytoplasm	3	Glucose-1-phosphate uridylyltransferase	39	89	10
37	20647	5.2	33.4	<i>galU</i>	Cytoplasm	8.3	UTP-glucose-1-phosphate uridylyltransferase	30	56	8
24	20463	5.7	39.8	<i>rffG</i>	Cytoplasm	5.1	dTDP-glucose 4,6-dehydratase	31	86	9
23/28	19487	5.8	43.4	<i>ugd</i>	Cytoplasm	5	UDP-glucose dehydrogenase	21/54	65/282	6/22
Regulation										
51	16313	5.2	67.5	<i>bipA</i>	Cytoplasm	1.5	Ribosome-binding GTPase	29	77	16
Detoxification										
20	17551	5.8	22.4	No name	Cytoplasm	1.9	Alkyl hydroperoxide reductase	32	63	7
Amino acid metabolism										
11	18183	6.1	45.3	<i>glyA</i>	Cytoplasm	4.5	Serine hydroxymethyl transferase	29	66	14
Lipid metabolism										
4	17046	6.7	49.3	<i>accC</i>	Cytoplasm	2.2	Biotin carboxylase	27	51	14
39	19707	5.9	19.1	<i>fabA</i>	Cytoplasm	0.58	β -Hydroxydecanoyl thioester dehydratase	27	69	5
35	19259	5.4	28.2	<i>fabI</i>	Cytoplasm	0.47	Enoyl-ACP reductase	27	85	7
Nucleotide metabolism										
22	15203	5.8	47.3	<i>purA</i>	Cytoplasm	4.4	Adenylosuccinate synthase	18	75	11
30	17763	5.9	51.5	<i>purB</i>	Cytoplasm	8.3	Adenylosuccinate lyase	38	173	19
Carbohydrate metabolism										
52	19243	5.5	99.2	<i>aceE</i>	Cytoplasm	1.1	Subunit of pyruvate dehydrogenase	26	124	20
31	15181	5.6	39.1	<i>fbaA</i>	Cytoplasm	4.7	Fructose-bisphosphate aldolase class II	20	50	7
13	18196	6.6	44.4	<i>ganA</i>	Periplasm	4.4	Endogalactanase	34	95	15
5	15062	6.5	56.7	<i>glpD</i>	Cytoplasm	5	Glycerol-3-phosphate dehydrogenase	38	174	25
21	20398	5.8	56	<i>glpK</i>	Cytoplasm	3.3	Glycerol kinase	29	223	17
14	15029	6.1	40.8	<i>glpQ</i>	Cytoplasm	2.8	Glycerophosphoryl diester phosphodiesterase	50	96	18
12	16958	6.2	48.1	<i>gltA</i>	Cytoplasm	2.6	Citrate synthase	18	67	9
55	46981	5.3	51.5	<i>gnd</i>	Cytoplasm	1.9	6-Phosphogluconate dehydrogenase	23	55	10
38	20551	5.7	28.6	<i>gpmA</i>	Cytoplasm	2.9	Phosphoglyceromutase 1	22	53	6
27	17827	5.9	61.2	<i>pgi</i>	Cytoplasm	5.6	Phosphoglucose isomerase	30	61	13
50	19893	5.1	87.2	<i>ppsA</i>	Cytoplasm	0.59	Phosphoenolpyruvate synthase	22	86	15
53	16955	5.7	64.5	<i>sdhA</i>	Cytoplasm	3.9	Succinate dehydrogenase	26	114	17
26	20198	5.3	62.9	<i>sfcA</i>	Cytoplasm	0.42	Malate dehydrogenase (gluconeogenesis)	26	101	13
54	15185	5.9	71.9	<i>tktA</i>	Cytoplasm	4.2	Transketolase 1 isoenzyme	21	107	14
43	20276	5.3	40	<i>yiaY</i>	Cytoplasm	0.3	Alcohol dehydrogenase	54	66	19

Table 1. cont.

Spot no.* and function	Accession no.†	pI	Mass (kDa)‡	Gene†	Location	Ratio‡	Putative function†	Coverage	Score§	No. of peptides matched
Protein folding and degradation										
42	19559	5.4	46.7	<i>clpX</i>	Cytoplasm	3	Subunit of ClpXP protease	32	116	12
10	15693	7.7	47.9	<i>degQ</i>	Periplasm	2.7	Serine endoprotease	34	181	15
36/56	15461	6.1	29.9	<i>fkpA</i>	Periplasm	11	FKPB chaperone	37/39	67/93	12/13
48	18669	4.9	57.5	<i>groL</i>	Cytoplasm	1.2	GroEL subunit of GroES/GroEL chaperone	57	118	40
40	18667	5.7	10.5	<i>groS</i>	Cytoplasm	2.7	GroES subunit of GroES/GroEL chaperone	55	71	5
6	16590	6.4	54.8	<i>pepA</i>	Cytoplasm	4.9	Aminopeptidase	17	55	9
29	20133	5.9	46.9	<i>pepB</i>	Cytoplasm	8.4	Aminopeptidase	37	154	18
9	17747	6.1	50.4	<i>pepQ</i>	Cytoplasm	0.77	Proline dipeptidase	18	53	7
47	20523	4.9	48.2	<i>tig</i>	Cytoplasm	3.4	Trigger factor	62	74	34
Transport										
46	14785	5	50.3	<i>atpD</i>	Cytoplasm	0.7	ATP synthase β subunit	37	76	11
2	18320	8.6	34.1	<i>gltI</i>	Periplasm	1.8	Glutamate-binding protein	61	149	21
34	18377	6.5	35.8	<i>mglB</i>	Periplasm	2.4	Galactose-binding protein	56	134	16
8	20639	6.4	61.3	<i>oppA</i>	Periplasm	3	Oligopeptide-binding protein	38	105	22
16	19819	7.6	41	<i>potF</i>	Periplasm	1.5	Putrescine-binding protein	22	59	8
3	20233	8.8	36.8	Unknown	Periplasm	2.9	Iron-binding protein	30	61	11
Transcription										
44/45	16922	5	36.6	<i>rpoA</i>	Cytoplasm	2	RNA polymerase α subunit	38/40	75/59	11/17
Translation										
49	46566	4.9	61.3	<i>rpsA</i>	Cytoplasm	1.6	S1 protein of 30S ribosomal subunit	55	210	29
15	20829	6.1	37.7	<i>trpS</i>	Cytoplasm	2.5	Tryptophanyl-tRNA synthetase	21	102	10
Unknown function										
17	15570	7.8	45.5	<i>ydjJ</i>	Unknown	3.5	Unknown	25	108	10
41	15573	8.5	17.1	Unknown	Unknown	9.1	Unknown	48	103	8
7	47171	6.3	50.7	Unknown	Unknown	2.9	Unknown	18	65	10
19	47181	6.6	25.6	Unknown	Unknown	0.42	Unknown	42	69	9

*Spot numbers refer to the proteins labelled in Fig. 2.

†As given in the *E. chrysanthemi* database (<http://asap.ahabs.wisc.edu/asap/ASAP1.htm>).

‡Spot intensity for each protein derived from the *opgG* mutant cells with respect to the protein derived from the wild-type cells.

§Score is $(-10)\log P$; P is the probability that the observed match is a random event.

||FkpA was detected in the *opgG* mutant strain NFB3500 only.

and 0.11 mg ml⁻¹ for the wild-type and the mutant, respectively.

Identification of proteins with an altered pattern of synthesis

The properties of several Opg⁻ phenotypes suggested that envelope perturbation had occurred. To quantify the amount of envelope perturbation, 1D SDS-PAGE of membrane proteins was performed. No significant difference was observed between the protein patterns for the *opgG* mutant and the wild-type, indicating that the synthesis of major membrane proteins was only slightly affected in the *opgG* mutant strain (data not shown). Thus, to get better insight into the Opg⁻ phenotype, a 2D comparative analysis of the soluble proteome of the two strains was performed.

The soluble proteins of *E. chrysanthemi* extracted from the wild-type strain EC3937, and from the *opgG* strain NFB3500, were separated on high-resolution 2D electrophoresis gels (pH 3–10) (Figs 1 and 2). Most of the spots observed were located in a pI range of 4.6–6.6. Three analytical gels for each condition were analysed and compared by the Melanie 5 software, which generates standardized synthetic images. The two resulting synthetic gels allowed detection of 275 and 218 spots for the wild-type and the *opgG* mutant, respectively. Spots of 55 polypeptides, showing differences in intensity between the wild-type and the mutant, were excised from the gels, submitted to trypsin digestion and analysed by MALDI-TOF MS. The experimental mass fingerprint was compared with the genomic database of *E. chrysanthemi* using Mascot software. For each polypeptide, the mass and pI obtained from the 2D analytical gels were in agreement with the theoretical mass and pI of the deduced protein. Functions were assigned to 51 of the 55 spots (Table 1). As expected, most of the proteins (42) originated from the cytoplasm, while 9 originated from the periplasm. The proteins were assigned into 11 classes of cellular function, with almost half (25) being assigned to protein folding and degradation and carbohydrate metabolism. We focused on proteins showing a differential expression level of around 2 or more and, among these, only three were expressed to a lesser extent in the mutant strain NFB3500.

Envelope composition is modified in *opgG* cells

In *E. chrysanthemi*, as in other Gram-negative bacteria, one of the phenotypes encountered in *opg* mutants is overproduction of the exopolysaccharides known to protect cells from environmental injury (Bohin, 2000). We report here the overexpression of five genes needed for exopolysaccharide biosynthesis. *cpsG* encodes a phosphomannomutase (Stevenson *et al.*, 1996). *galE*, *galU* and *galF* encode proteins that catalyse steps in LPS core biosynthesis. GalE is the UDP-glucose 4-epimerase, and GalU and GalF are the two subunits of the UTP-glucose-1-phosphate uridylyltransferase catalysing the reversible reaction of UTP and glucose 1-phosphate into UDP-glucose. Finally, *ugd* encodes

UDP-glucose dehydratase and, in addition, it participates in a modification of the core of LPS, which confers resistance toward antimicrobial peptides in *Salmonella enterica* and *Yersinia pestis* (Mouslim & Groisman, 2003; Winfield *et al.*, 2005). Furthermore, RffG production was increased; this protein is a dTDP-glucose 4,6-dehydratase needed for the synthesis of both O antigen and enterobacterial common antigen (Keseler *et al.*, 2005). An increase in LPS biosynthesis has already been shown in a comparative quantification of monosaccharide production between the *opgG* and wild-type *E. chrysanthemi* strains. The analysis revealed a 1.5-fold increase in the level of Kdo, which is the specific monosaccharide of the core of the LPS, and in the level of N-acetylglucosamine, which is part of the lipid A moiety of LPS, in the *opgG* mutant strain, while the peptidoglycan level remained stable (Bohin *et al.*, 2005).

Lipid metabolism was enhanced and fatty acid saturation was decreased in *opgG* cells. Synthesis of a subunit of the acetyl CoA carboxylase (AccC protein), which participates in the first step of fatty acid synthesis, was increased, while synthesis of the enoyl-ACP reductase (FabI protein), which is implicated in the biosynthesis of unsaturated fatty acids, was reduced. Biochemical identification and quantification of the fatty acid composition of the wild-type and the *opgG* mutant of *E. chrysanthemi* EC3937 revealed a 20–30% reduction in the amount of C_{16:1} and C_{18:1} unsaturated fatty acids in the membrane phospholipids of the *opgG* mutant cells (Bohin *et al.*, 2005). Furthermore, expression of enzymes controlling glycerol catabolism (GlpD, GlpQ and GlpK) was increased. These findings suggest a rearrangement of the envelope structure in the *opgG* mutant cells.

Catabolic enzyme synthesis is enhanced in *opgG* cells

In this work, transport systems were detected by their soluble periplasmic-binding-protein component (Table 1), since all of these components belong to the ABC transporter family of proteins. Increased synthesis of two proteins, which are components of the nutrient uptake system, was detected: MglB, a galactose-binding protein; and OppA, the oligopeptide-binding protein serving for nutrition by peptides and recycling of muropeptides (Monnet, 2003). In addition, synthesis of a periplasmic endogalactanase (GanA) was increased. The gene encoding endogalactanase belongs to an operon controlling galactan uptake (A. Delangle, A.-F. Prouvost, V. Cogez, J.-P. Bohin, J.-M. Lacroix & N. Hugouvieux-Cotte-Pattat, unpublished results). Nutrient uptake appeared to be stimulated in the *opgG* mutant.

Enzymes related to carbohydrate metabolism were detected. All of the metabolic pathways discussed are according to Keseler *et al.* (2005). A gene from one of the pathways, *sfcA*, encoding the malic enzyme, was expressed to a lesser extent in the mutant. This enzyme catalyses the transformation of malate to pyruvate in one of the two possible gluconeogenesis pathways. Three overexpressed

genes encoded enzymes that are part of the glycolytic pathway. Phosphoglucose isomerase (Pgi) catalyses the first step of glucose degradation. The major fructose-bisphosphate aldolase (FbaA) displays 95 % of its activity in glycolysis and is not required for gluconeogenesis in *Escherichia coli*. Synthesis of the phosphoglycerate mutase (encoded by *gpmA*) is increased in other bacterial species under various environmental stress conditions (Sanchez *et al.*, 2005). In addition, synthesis of the three enzymes of glycerol catabolism (GlpD, GlpQ and GlpK), which catalyse conversion of glycerol into dihydroxyacetone phosphate, was increased. The *galE*, *galU* and *galF* genes, whose products are needed for polysaccharide synthesis and also for galactose degradation, were overexpressed. Furthermore, increased levels of two proteins catalysing steps in the pentose phosphate pathway were observed: 6-phosphogluconate dehydrogenase (encoded by *gnd*) and the major transketolase (encoded by *tktA*). In *E. coli*, transketolase is the key enzyme regulating the flux between the pentose phosphate pathway and glycolysis. Transketolase is essential for pentose catabolism (Romeo & Snoep, 2005); increased synthesis leads to an increase in glyceraldehyde 3-phosphate and fructose 6-phosphate, thus supplying glycolysis with intermediates at two different levels. In addition, transketolase plays a protective role during oxidative stress in *Saccharomyces cerevisiae* (Slekar *et al.*, 1996) and its synthesis is increased in conditions of bile-salt stress in *Bifidobacterium longum* (Sanchez *et al.*, 2005). Two genes encoding enzymes of the tricarboxylic acid (TCA) cycle were overexpressed: *gltA* and *sdhA*, encoding citrate synthase and succinate dehydrogenase respectively. Increased synthesis of the latter enzyme suggested that the TCA cycle was closed, and functioned for both precursor and energy synthesis. In *E. coli* under aerobic conditions, the TCA cycle was not closed, stop before succinate, and was almost exclusively dedicated to precursor synthesis (Romeo & Snoep, 2005). Data indicate that in *E. coli*, an increase in energy requirement due to temperature-induced production of recombinant proteins is associated with succinate dehydrogenase overexpression and an increase in respiration (Hoffmann *et al.*, 2002). This suggests that energy metabolism is stimulated in response to OPG deficiency.

Protein folding and degradation are stimulated in *opgG* cells

It is well documented that the stress response includes increased synthesis of three classes of proteins: chaperones, proteases and peptidases. Synthesis of seven proteins governing protein folding or degradation was increased in the *opgG* mutant (Table 1). FkpA, a periplasmic chaperone with a *cis-trans* prolyl isomerase activity (Justice *et al.*, 2005), was detected in the proteome of the *opgG* mutant strain only. In *E. coli*, the *fkpA* gene is activated by an increase in unfolded envelope proteins caused by stress conditions affecting envelope integrity (Dartigalongue *et al.*, 2001). FkpA has been implicated in the heat-shock response and intracellular survival of *S. enterica* sv. Typhimurium (Horne *et al.*, 1997).

Intracellular survival depends on the Mip protein, which is an FkpA-like protein in *Legionella pneumophila* (Cianciotto *et al.*, 1990). Five other proteins were located in the cytoplasm. Tig is a chaperone with *cis-trans* prolyl isomerase activity that is bound to the ribosome and cooperates with the DnaK chaperone to promote folding of newly synthesized proteins (Deuerling *et al.*, 1999). For GroEL/GroES, another chaperone, synthesis of the regulatory component GroES was found to be significantly increased (Table 1). DegQ is a periplasmic serine endoprotease that degrades denatured or unfolded proteins that are produced in normal growth conditions, or are accumulated after various stress conditions (Yohannes *et al.*, 2004; Kolmar *et al.*, 1996). ClpX, the ATPase subunit of the ClpXP protease, was overexpressed, while ClpP, the catalytic subunit of this protease, was not detected. It is possible that we were not able to detect ClpP, or, alternatively, that only the *clpX* gene was overexpressed. The *pepA* and *pepB* genes, encoding two cytoplasmic peptidases, were overexpressed. In addition to its peptidase activity, PepA is involved in the regulation of virulence factors in *Vibrio cholerae* (Behari *et al.*, 2001). Furthermore, it has been reported that OppA and MglB, in addition to their role in substrate uptake, could play a role in protein folding. These chaperone-like proteins may participate in protection from stress in the periplasm (Richarme & Caldas, 1997). The results presented here suggest that *E. chrysanthemi* *opgG* strain responds to OPG deficiency by increasing the expression of several stress-response proteins.

Concluding remarks

The present work constitutes what we believe to be the first proteomic analysis of the production of soluble proteins in an *opg* mutant. Despite the global and non-exhaustive character of this analysis, it confirms the phenotypes previously described, and reveals several new phenotypes. *E. chrysanthemi* responds to OPG deficiency by activating cellular processes known to protect the cell against many environmental stresses, suggesting that the *opgG* strain is impaired in the perception of its environment. Our data demonstrate that the non-virulent character of *opgG* mutants does not result simply from modification in envelope composition leading to an impaired interaction between the surfaces of the bacteria and its host cell. Lack of infectivity is also a result of alterations in metabolism and fitness of *opgG* cells.

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