Effect of potato suberin on *Streptomyces scabies* proteome

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SUMMARY

Two-dimensional (2D) PAGE was used to detect proteins induced in *Streptomyces scabies* by potato suberin, a lipidic plant polymer. Nineteen up-regulated proteins were excised from 2D gels and analysed by N-terminal sequencing or tandem mass spectrometry (MS/MS). Four of the up-regulated proteins could be linked to the bacterial response to stress (AldH, GroES, TerD and LexA). Specific metabolic pathways seemed to be activated in the presence of suberin, as shown by the increased expression of specific transporters and of enzymes related not only to glycolysis, but also to nucleotide and amino acid metabolism. Suberin also appeared to influence secondary metabolism as it also caused the overproduction of the BldK proteins that are known to be involved in differentiation and secondary metabolism.

INTRODUCTION

Streptomyces are Gram-positive, filamentous bacteria that are known for their ability to produce a wide variety of both extracellular hydrolytic enzymes and active secondary metabolites. Their genome is characterized by a high G+C content. Most members of this genus are soil-inhabiting saprophytes. However, some of them are plant pathogens and cause common potato scab, an economically important plant disease found in Canada and in most potato-growing areas in the world (Hill and Lazarovits, 2005). *Streptomyces scabies* is the main causal agent of common potato scab (Lambert and Loria, 1989a) although other species such as *Streptomyces acidiscabies* (Lambert and Loria, 1989b), *S. turgidiscabies* (Miyajima *et al.*, 1998), *S. europaeiscabiei*, *S. stelliscabiei* (Boucek-Mechiche *et al.*, 1998), *S. luridiscabiei*, *S. niveiscabiei* and *S. puniscabiei* (Park *et al.*, 2003) are widespread in North America, Asia and Europe.

The virulence mechanisms associated with these pathogens include the secretion of nitro-aromatic compounds called thaxtomins that exhibit phytotoxic properties. Thaxtomins are

4-nitroindol-3-yl-containing 2,5 dioxopiperazines (King *et al.*, 1989). Thaxtomin A is the most abundant toxin produced by *S. scabies* and appears to be essential to symptom development. Purified thaxtomin A reproduces symptoms of common scab when applied on aseptic potato tubers (Lawrence *et al.*, 1990). Disruption mutants of the thaxtomin biosynthesis genes become avirulent on potato tubers (Healy *et al.*, 2000).

Thaxtomin A is synthesized by a peptide synthetase encoded by the *txtA* and *txtB* genes (Healy *et al.*, 2000). Hydroxylation of the cyclic dipeptide is catalysed by a cytochrome P450 monooxygenase encoded by *txtC* (Healy *et al.*, 2002), while nitration of the dipeptide is carried out by an enzyme similar to mammalian nitric oxide synthase (Kers *et al.*, 2004). The genes responsible for thaxtomin biosynthesis are located on a pathogenicity island found in the genome of *S. scabies*, *S. acidiscabies* and *S. turgidiscabies* (Bukhalid *et al.*, 2002).

Bacteria commonly detect specific plant metabolites, which they use as signals to regulate the expression of virulence genes that allow their adaptation to the plant environment. For example, in *Agrobacterium tumefaciens*, several virulence genes are activated by acetosyringone and other phenolic molecules produced by the plant (Melchers *et al.*, 1989). It has been shown that cellobiose can induce thaxtomin A production in *S. acidiscabies* (Wach *et al.*, 2007). However, cellobiose by itself induces only poorly the dipeptide synthesis in *S. scabies* strains such as EF-35. Nevertheless, the disaccharide did increase thaxtomin A production when added to a culture medium containing suberin (Lerat *S. et al.*, unpublished data). Addition of suberin in a minimal medium is sufficient to allow good thaxtomin A production in *S. scabies* strains (Beausejour *et al.*, 1999). Production of this toxin varied between 10 and 250 µg per g of cells in the presence of suberin depending on *S. scabies* strains (Beausejour *et al.*, 1999).

Suberin is a complex polymer consisting of both polyaromatic and polyaliphatic domains (Kolattukudy *et al.*, 1981). Potato suberin was described as consisting of a hydroxycinnamic acid-monomer lignol polyphenolic domain embedded in the primary cell wall, and covalently linked to a glycerol-based polyaliphatic domain located between the primary cell wall and the plasma membrane (Bernards, 2002).

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The lipidic polymer provides effective resistance to dehydration and plays an important role in the protection of potato tubers by acting as a physical barrier against most pathogenic microorganisms (Kolattukudy, 1984). However, *S. scabies* can overcome this defence mechanism by producing a heat-stable esterase that breaks down suberin (McQueen and Schottel, 1987).

Differential protein display by two-dimensional (2D) gel electrophoresis and proteome analysis is a powerful tool to monitor changes occurring in the protein expression of an organism (Wilkins *et al.*, 1996). Analysis of streptomycete proteomes has been carried out to study bacterial development (Hesketh *et al.*, 2002; Kim *et al.*, 2005a,b; Orsaria *et al.*, 1998; Piette *et al.*, 2005; Vohradsky and Thompson, 2006), secondary metabolite production (Hesketh and Chater, 2003; Lai *et al.*, 2002; Mohrle *et al.*, 1995; Wang and Yuan, 2005) and mechanisms involved during interaction with a plant (Langlois *et al.*, 2003). Proteomic studies have also been used to study plant–microbe interactions involving bacteria such as *Streptomyces coelicolor* (Langlois *et al.*, 2003), *Rhizobium leguminosarum* (Guerreiro *et al.*, 2000), *Pseudomonas putida* (Heim *et al.*, 2003) and *Sinorhizobium meliloti* (Natera *et al.*, 2000). *S. coelicolor* is a saprophytic microorganism that has been shown to colonize various plants including potato (Doubou *et al.*, 2001). Grown in the presence of plants, this species overproduced proteins involved in energetic metabolism, protein synthesis, degradation of amino acids, alkenes, cellulose, tellurite resistance and growth under general physiological or oxidative stress conditions (Langlois *et al.*, 2003).

The aim of the present study was to elucidate the physiological adaptations induced in *S. scabies* by the presence of the complex plant polymer suberin. 2D gel electrophoresis was used to identify newly expressed proteins from *S. scabies* in the presence of the polymer. Edman sequencing and mass spectrometry were used to identify selected proteins.

RESULTS AND DISCUSSION

Comparison of protein profiles in MM and SM

Proteomes of *S. scabies* grown with or without suberin were compared. Growth curves were similar in both conditions (data not shown), suggesting that changes observed in the proteomes could be directly linked to the addition of the plant polymer. Soluble proteins were extracted from 40-h-old cultures which represent the early stationary phase of the life cycle. Thaxtomin A, a pathogenicity factor produced by *S. scabies* in the suberin medium (SM), became detectable after about 36 h of growth under the conditions used in this study. Furthermore, the suberin-inducible esterase that is thought to be involved in the polymer degradation also became detectable in the supernatant after about 36 h of growth (Beausejour *et al.*, 1999).

Fig. 1 Proteome map of *Streptomyces scabies* soluble internal proteins visualized with silver stain. Proteins were separated in the first dimension over a pH range of 4.0–7.0. *S. scabies* was grown in minimal medium. The region corresponding to pH 5.5–6.7 shown in Fig. 2 is boxed.

Image analysis of the *S. scabies* proteome in minimal medium (MM) revealed over 650 protein spots in the pH range 4.0–7.0 and the size range 10–180 kDa (Fig. 1). Narrow-range immobilized pH gradient (IPG) strips (pH 4.0–5.0, 4.5–5.5, 5.0–6.0 and 5.5–6.7) were used to further resolve these proteins. Thirty protein spots were detected only in the presence of suberin in at least three independent experiments. These spots were excised from the gels and subjected to Edman degradation or MS analysis for protein identification.

Seven spots could not be conclusively assigned to a protein identification. Four spots were found to contain more than one protein and were not further analysed. Amino acid sequences were obtained for 19 spots either by N-terminal sequencing or mass spectrometry analysis (MS/MS). Four amino acid sequences were N-terminal sequences of *S. scabies* protein spot and the 15 other amino acid sequences were deduced by MS/MS analysis and database searches (Table 1). Ten of the sequenced proteins were excised from the pH 5.5–6.7 gels, seven from the pH 5–6 gels and one each from the pH 4.0–5.0 gels and 4.5–5.5 gels.

Figure 2 presents the proteome associated with gels covering pH 5.5–6.7. A search for sequence homology with the NCBI database allowed the identification of most of the proteins with a high level of confidence (Table 1), although the annotation of the *S. scabies* genome sequence was not yet available in the public database. This indicates the relevance of proteome analysis even involving organisms for which the genome sequence is not available or is not yet annotated. The Mascot score associated with the protein identified by mass spectrometry varied between 61 and 533.

The presence of a region corresponding to the amino acid sequences obtained by Edman analysis or MS was confirmed in the *S. scabies* genome (Table 2). Blast analysis using the complete amino acid sequence deduced from the corresponding *S. scabies* gene allowed a more precise identification of some of

Table 1 Identification of protein spots up-regulated in the presence of suberin.

Protein designation	pI*	MW (kDa)*	Amino acid sequences deduced by MS/MS analysis and database search†‡	Matching protein	
				Probable function	Bacterium
1	5.6	67.6	AVALEAR—IGTPALTTR—RPAFQDYAQR—NVDTVESLAAEHAR—QAEAAALDSGIVTNR—GLGTAEMDEVAGLIDR—NAIPADPNGAWYTSGIR—SYGTDPATGLIDYEALR—VLTGDFDPVPHAQIVTTTHK	serine hydroxymethyltransferase	<i>S. coelicolor</i>
2	5.8	58.1	RYGVEIK—GAEVVVVGR—DCAQVGIASIQR—AQLLVNVVEAAER—GVTIGRPMPLLLTR—HRDCAQVGIASIQR—ELNEDPACTGYIVQLPLPK—LVLNEPAPLPCTPNGVLTLLR—ILELMDPKDADGLHPMNLGR	p methylenetetrahydrofolate dehydrogenase and cyclohydrolase	<i>S. avermitilis</i>
4	5.88	64.9	MLAGLEDVNGGAIR	ABC transporter ATP-binding protein	<i>S. coelicolor</i>
5	5.98	63.7	QIAEPMK—TLGIVGESGSGK—VDDYPHQFSGGMR	BldKD	<i>S. coelicolor</i>
10	6.2	75.2	HLPDSVLETPDAK—GKHLPSVLETPDAK	p ABC transporter protein	<i>S. avermitilis</i>
12	6.27	63.2	LRGVELLAR—GASVVHAPALR—RADELGALLQR—IVPLADDSSELLAATK—ALPGAGRDEHAVETAMAR	p transcriptional regulator	<i>S. avermitilis</i>
13	6.22	49.2	RVIEVIRDSVQR	LexA protein	<i>S. lipmanii</i>
16	6.46	48.7	GKHLPSVLETPDAK	p ABC transporter protein	<i>S. avermitilis</i>
17	5.85	70.7	LLGIVTNR—DMAFETDR—GMGSLGAMQSR—GLITVKDFVK—VGVGPGSICTR—DGAQALIDAGVDGIK—LVPEGIEGQVPYR—SYRGMGSLGAMQS—ESHPHDIQMTVEAPNYSR	inosine-5-monophosphate dehydrogenase	<i>S. coelicolor</i>
18	6.1	56.1	TLPIAGHRVEIR—RADELGALLQRR—ALPGAGRDEHAVETAMAR	p transcriptional regulator	<i>S. avermitilis</i>
25	5.58	45	SYFR—KVFVIDDK—KFVADYK	p periplasmic branched chain amino acid binding protein	<i>S. coelicolor</i>
29	5.75	44	AARAQTAWAALAPGDR—DLLDYAAGGVER—ELGPDALTAFTETK—ILVQR—PAETTPLTALR—QVAAKGAALLK—VGDPADESTEMGPLISR—WEAGNVR—YWTFFGGFK	p aldehyde dehydrogenase	<i>S. coelicolor</i>
31	5.73	34	LAFALADK—SVQTLVDGDK—IQYGGSVK	triose-phosphate isomerase	<i>S. coelicolor</i>
43	4.77	41.2	GYVSAVQNK—LEMELTVER—RTLSSIPGAAVTSIR—LIAQRPSLTVEEVVDEFR—FVIEPLEPGFGYTLGNSLR—MLIAQRPSLTVEEVVDEFR—QGPGVLTAADIAPPAGVEVHPDLVLATLNGK	RNA polymerase alpha subunit	<i>S. coelicolor</i>
50	4.55	35.2	LVDTVGGGANFVQNAILGPLEDK	hypothetical protein	<i>S. avermitilis</i>
N-terminal sequence from <i>S. scabiei</i> protein spot					
21	5.82	13.5	LVIPDTACEPPQFGVL	GroES	<i>S. coelicolor</i>
24	5.29	38	SAPAXDEDITIVMGVNDQXYDAAN	G-3-P dehydrogenase	<i>S. coelicolor</i>
30	5.74	41	PIATPEVYNIQLD	fructose-biphosphate aldolase	<i>S. galbus</i>
47	4.28	40	IVFPVSIHDAENR	TerD	<i>S. coelicolor</i>

*Experimental value as determined by gel image analysis.

†MS/MS: analysis by Quadrupole ion trap mass spectrometry and interpretation using the SEQUEST algorithm or the MASCOT algorithm.

‡Oligopeptides are separated by an indent.

the proteins. Although data derived from MS analysis indicated that proteins 4, 5, 10 and 16 could be classified as ABC transporter proteins, sequence analysis of the entire corresponding genes in the *S. scabiei* genome suggested that protein 4 was involved in cellobiose and maltose transport, while proteins 5, 10 and 16 were part of the BldK oligopeptide transport system. Furthermore, MS analysis suggested that spot 29 shared an amino acid domain with acetaldehyde dehydrogenases. The blast analysis, using the corresponding gene of the *S. scabiei* genome, suggested that the substrate of this enzyme was specifically phenylacetaldehyde. However, the results of the two analyses were not always in agreement. In the case of spots 12 and 18, the amino acid sequences obtained by MS analysis suggested that these proteins

were transcriptional regulators. However, the complete amino acid sequence deduced from the corresponding *S. scabiei* gene shared 93% similarity with the amino acid sequence of *S. avermitilis* uroporphyrinogen-111 synthase.

Following these analyses, it was possible to divide *S. scabiei* up-regulated proteins into six groups according to their function: proteins involved in amino acid metabolism (proteins 1, 2, 25), proteins involved in nucleotide and nucleic acid metabolism (proteins 17, 43), proteins involved in heme metabolism (proteins 12, 18), proteins involved in carbon acquisition (proteins 4, 29), proteins linked to the glycolytic pathway (proteins 24, 30, 31), and finally stress-induced proteins and proteins involved in secondary metabolism (proteins 5, 10, 13, 16, 21, 47). The function

Fig. 2 Comparative two-dimensional gel electrophoresis of soluble internal proteins of *Streptomyces scabies* stained with Coomassie Brilliant Blue R-250. The large gel images represent the MM and SM proteomes using IPG strips pH 5.5–6.7 (panels A and B, respectively). Regions of interest are boxed (I–VI). Identified proteins are circled and identified on the excerpts from the SM proteome.

of protein 50 could not be determined based on amino acid or nucleotide sequences, but appeared to possess a signal peptide (Analysis with the SignalP 3.0 software using a hidden Markov model, signal peptide probability: 1).

Analysis of spots 10 and 16 as well as spots 12 and 18 yielded the same protein identification even though they exhibited a slightly different molecular weight or pI. This suggests that uroporphyrinogen-III synthase and BldKB could be postranscriptionally modified. In *S. coelicolor*, Hesketh *et al.* (2002) have estimated a mean of 1.2 protein isoforms per gene.

Enzymes involved in the glycolytic pathway or in carbon acquisition are up-regulated in the presence of suberin

The presence of suberin in *S. scabies* culture media seemed to induce enzymes involved in the energy metabolism of the bacteria. Three enzymes associated with glycolysis, glyceraldehyde-3-phosphate dehydrogenase, fructose-bis-phosphate aldolase and triose-phosphate isomerase, were up-regulated in the presence of suberin in this study. The up-regulation of these glycolytic enzymes might be attributed to the presence of glycerol in suberin as glycerol is an important constituent of suberin as demonstrated by Moire *et al.* (1999). To be catabolized, glycerol is phosphorylated and then oxidized to dihydroxyacetone phosphate by a glycerol phosphate dehydrogenase. This last product, as well as glyceraldehyde-3-phosphate, is the substrate for the three enzymes of the glycolytic pathway identified here. The fact that suberin has also been shown to induce the expression of a gene encoding a glycerol phosphate dehydrogenase in *S. scabies* (A. Lauzier and C. Beaulieu, unpublished data) also provided evidence that suberin constituted a source of glycerol for the pathogenic bacteria.

Serine hydroxymethyl-transferase and methylene-tetrahydrofolate dehydrogenase (Table 2, proteins 1 and 2) are two other enzymes

that were up-regulated when *S. scabies* was grown in the presence of suberin. These enzymes are involved in the biosynthesis of glycine and serine, respectively (Stryer, 1995). Phosphoglycerate, a metabolite of the glycolytic pathway, is the biosynthetic precursor of serine and glycine. The up-regulation of both methylene-tetrahydrofolate dehydrogenase and serine hydroxymethyl-transferase could thus be a consequence of the up-regulation of several enzymes associated with glycolysis.

Bernards and Lewis (1998) established that alkaline hydrolysis of suberized potato wound periderm tissues released cell-wall-bound aldehydes including vanillin. This correlates with the up-regulation of aldehyde dehydrogenase in the proteome of *S. scabies* grown in the presence of suberin (Table 1, protein 29). These results suggest that the bacterium could produce extracellular enzymes that attack suberin to release aldehydes. In support of this hypothesis, a proteomic differential display technique used to study the cellular responses of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* exposed to vanillin revealed that enzymes involved in glycolysis, in heme biosynthesis and in cellobiose catabolism were activated in the presence of the aldehyde (Shimizu *et al.*, 2005). These same types of enzymes were activated in this study by the presence of suberin, suggesting that aldehydes, associated with suberin or lignin, might induce similar physiological responses. The amplified production of enzymes involved in heme synthesis, such as uroporphyrinogen III synthase (Table 2, proteins 12/18), might reflect an increased need for this cofactor in microorganisms exposed to suberin or lignin. This idea is supported by the observation that several heme-containing enzymes participate in the degradation of phenolics and aldehydes associated with plant polymers (Shimizu *et al.*, 2005).

Another example of the link between carbohydrate metabolism and phytochemicals is the unexplained activation of cellobiose catabolism in *P. chrysosporium* by exposure to vanillin (Shimizu

Table 2 Identification of *S. scabiei* genes containing the translated amino acid sequences of proteins up-regulated in the presence of suberin.

Protein designation	Gene position in the genome of <i>S. scabiei</i> *	Matching protein			Protein similarity (%)‡
		Probable function	Bacterium	Accession no.†	
1	4 021 189 (482)	serine hydroxymethyltransferase	<i>S. coelicolor</i> A3(2)	NP_628992.1	97
2	4 030 061 (284)	p methylenetetrahydrofolate dehydrogenase and methylenetetrahydrofolate cyclohydrolase	<i>S. avermitilis</i> MA-4680	NP_824619.1	99
4	5 589 110 (379)	ABC transporter ATP-binding protein assisting in cellobiose and maltose transport (MsiK)	<i>S. reticuli</i>	CAA70125.1	97
5	3 570 317 (353)	BldKD, ABC transporter intracellular ATPase subunit	<i>S. coelicolor</i> A3(2)	NP_629265.1	85
10/16	3 572 493 (600)	BldKB, ABC transport system lipoprotein	<i>S. coelicolor</i> A3(2)	NP_629263.1	73
12/18	6 234 465 (385)	uroporphyrinogen-III synthase	<i>S. avermitilis</i> MA-4680	NP_826297.1	93
13	2 789 747 (259)	LexA repressor	<i>S. avermitilis</i> MA-4680	NP_823639.1	99
17	4 097 422 (500)	inosine-5-monophosphate dehydrogenase	<i>S. coelicolor</i> A3(2)	NP_628928.1	97
21	4 106 605 (102)	10-kDa chaperonin (Protein Cpn10) (GroES protein)	<i>S. albus</i> G	Q00769	98
24	7 724 061 (336)	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	<i>S. clavuligeris</i>	ABA26666.1	96
25	7 633 671 (409)	branched chain amino acid binding protein	<i>S. coelicolor</i> A3(2)	NP_626269.1	91
29	8 171 015 (458)	phenylacetaldehyde dehydrogenase	<i>S. avermitilis</i> MA-4680	NP_827902.1	90
30	4 748 812 (340)	fructose-bisphosphate aldolase	<i>S. avermitilis</i> MA-4680	NP_825700.1	96
31	7 726 447 (290)	triosephosphate isomerase	<i>S. avermitilis</i> MA-4680	NP_827474.1	83
43	4 144 299 (340)	DNA-directed RNA polymerase alpha subunit	<i>S. avermitilis</i> MA-4680S. <i>coelicolor</i> A3(2)	NP_826130.1 NP_628887.1	100
47	7 128 592 (191)	tellurium resistance protein	<i>S. avermitilis</i> MA-4680	NP_826981.1	94
50	8 216 882 (429)	hypothetical protein SAV6777	<i>S. avermitilis</i> MA-4680	NP_827953.1	54

*Protein-coding gene was predicted using the GeneMarkHMM program. The number of amino acids in the deduced protein is indicated in parentheses.

†Accession number in NCBI database.

‡Sequence comparison was determined using the Clustalw program.

et al., 2005). The identification in the present study of a cellobiose and maltose ABC transporter in the proteome of *S. scabiei* might simply reflect the enzymatic degradation of suberin by the bacteria that results in the release of aldehydes. However, in *S. acidiscabiei*, cellobiose plays an important role in pathogenicity by inducing the biosynthesis of thaxtomin A (Wach *et al.*, 2007). In other phytopathogenic organisms, several ABC transporters have been linked to virulence. Inactivation of ABC transporters in *Botrytis cinerea* (Schoonbeek *et al.*, 2001), *Fusarium culmorum* (Skov *et al.*, 2004), *Erwinia chrysanthemi* (Nachin *et al.*, 2001), *Mycosphaerella graminicola* (Stergiopoulos *et al.*, 2003), *Gibberella pulicaris* (Fleissner *et al.*, 2002) and *Magnaporthe grisea* (Urban *et al.*, 1999) have all been shown to have a direct effect on pathogenicity.

Stress-related proteins are up-regulated in the presence of suberin

Acetaldehyde dehydrogenases are general detoxifying enzymes that catalyse the oxidation of aldehydes to carboxylic acids (Liu and Schnable, 2002; Nakazono *et al.*, 2000; Sunkar *et al.*, 2003). Over-expression of aldehyde dehydrogenase genes in eukaryotic cells resulted in an increased resistance to oxidative stress

(Sunkar *et al.*, 2003; Yoon *et al.*, 2004). Aldehyde dehydrogenases are also involved in resistance to osmotic stress (Boch *et al.*, 1997) and in the response to iron deprivation in *Bacillus subtilis* and *Pseudomonas putida*, respectively (Heim *et al.*, 2003). Suberin medium appears to represent a stressful environment, given the induction of stress-related proteins such as GroES, an oligomeric molecular chaperone (Fink 1999); TerD, a homologue of a tellurite resistance protein (Hill *et al.*, 1993) in *Serratia marcescens*; LexA, a protein of the DNA repair SOS-like regulatory system (Little and Mount, 1982); as well as an acetaldehyde dehydrogenase (Table 2, proteins 21, 47, 13 and 29 respectively). This confirms the work of Douliez (2004) who previously demonstrated that suberin monomers were stressing agents and act as membrane perturbants. Similar conclusions can be drawn from the proteomic study that identified homologues of both GroES and TerD that were induced in *S. coelicolor* in the presence of plants (Langlois *et al.*, 2003). Furthermore, a putative tellurite resistance protein was also up-regulated in the actinomycete *Frankia* during the interaction between the symbiont and its host plant (Hammad *et al.*, 2001). It appears that actinomycetes which interact with plants had to adapt to physiological stresses caused by the plants despite their adaptation to acquire carbon and energy through degradation of plant material.

Suberin influences secondary metabolism

In streptomycetes, the onset of secondary metabolism and the transition phase from vegetative growth to the stationary phase depends on various molecular signals, including guanoside nucleotides (Chakraborty and Bibb, 1997), branched-chain amino acids (Sprusansky *et al.*, 2005) and extracellular oligopeptides recognized by BldK proteins (Nodwell *et al.*, 1996). This proteomic study revealed that the enzymes which are involved in the biosynthesis or the transport of these signal molecules were differentially produced in response to suberin.

The streptomycetes have evolved in order to tie the degree of expression of stationary phase genes to the availability of branched-chain amino acids. Degradation of these amino acids in Gram-positive bacteria supplies precursors for membrane synthesis during sporulation and polyketide antibiotic production (Molle *et al.*, 2003; Sprusansky *et al.*, 2005). As the expression of the transporter for branched-chain amino acids was up-regulated in the presence of suberin and as the main secondary metabolite of *S. scabiei* was produced in the presence of this polymer, suberin thus appeared to promote the secondary metabolism.

Among the other substances specifically produced in the presence of suberin, we found two BldK proteins. In streptomycetes, *bld* genes are involved in morphological differentiation (Nodwell *et al.*, 1999) as well as in secondary metabolite production (Champness, 1988; Elliot *et al.*, 2003; Merrick, 1976). Differential production of BldK proteins and thaxtomin biosynthesis represented strong evidence that suberin promoted the secondary metabolism. The genetic locus *bldK* was characterized as a gene cluster coding for an oligopeptide permease complex that acts as the first receptor of an extracellular oligopeptidyl signal (Nodwell *et al.*, 1996). Nodwell and Losick (1998) have purified an oligopeptide that recognizes the BldK complex which regulates *S. coelicolor* differentiation. In *S. scabiei*, little is known about the regulation of both the differentiation process and the biosynthesis of secondary metabolites.

It would have been expected that enzymes involved in thaxtomin biosynthesis would be overexpressed in the presence of suberin. However, no proteins involved in thaxtomin biosynthesis were identified in this study. This may have been due to their lack of abundance or it may simply have been due to the difficulty in resolving such high-molecular-weight proteins via 2D electrophoresis (Warren *et al.*, 2003).

In streptomycetes, the stringent response depends on an increase in the concentration of the hyperphosphorylated guanosine nucleotide, ppGpp (Inaoka *et al.*, 2003). ppGpp is an effector that modulates both RNA polymerase (Toulokionov *et al.*, 2001) and IMP dehydrogenase (Ochi *et al.*, 1982). These last enzymes were overproduced by *S. scabiei* in the presence of suberin (Table 2, proteins 17 and 43). IMP dehydrogenase, the first enzyme in the GMP synthesis pathway, is negatively regulated by

ppGpp (Ochi *et al.*, 1982). The up-regulation of IMP dehydrogenase synthesis caused by suberin suggests that the stringent response was stronger in the minimal medium. Biosynthesis of the secondary metabolite thaxtomin A in the presence of suberin thus did not appear to depend only on nutritional stress. The presence of plant extracts in *S. coelicolor* culture medium also delayed the onset of a stringent response. Although *S. coelicolor* overproduced several proteins involved in adaptation to physiological and oxidative stress in the presence of plants, repression of a homologue of Tig, a cell division trigger factor, was observed in this condition (Langlois *et al.*, 2003). This Tig factor was shown to be essential for growth under starvation conditions (Gothel *et al.*, 1998).

In *Rhizobium tropici*, IMP dehydrogenase was shown to be essential for effective nodulation (Ricciolo *et al.*, 2000). IMP dehydrogenase, which was also differentially produced by *S. scabiei* in minimal and suberin media, could thus also be an important factor for the interaction between the pathogenic bacteria and its host.

This study suggests that suberin is not only a physical barrier to *S. scabiei* penetration in plant tissues (Lulai and Corsini 1998) but that the polymer seems to affect the physiology of the pathogen profoundly. Suberin not only acts as a carbon source for the bacteria (Beausejour *et al.*, 1999), but also appears to generate physiological stress and to modulate the activity of elements linked to bacterial secondary metabolism.

EXPERIMENTAL PROCEDURES

Bacterial culture conditions

Inoculum of *S. scabiei* strain EF-35 (Faucher *et al.*, 1992) was prepared by growing the strain, with shaking (300 r.p.m.) in trypticase soy broth (TSB, Difco Laboratories, Detroit, MI) for 48 h at 30 °C. The culture was then centrifuged and resuspended in five volumes of fresh TSB. This suspension was used to inoculate starch minimal media (Beausejour *et al.*, 1999) supplemented (or not) with 0.2% potato suberin at a ratio of 1:60 (v/v). Suberin was extracted from potato tuber skin as previously described by Kolattukudy and Agrawal (1974). Bacteria were then incubated in this medium with shaking for 40 h at 30 °C. Before proceeding to the protein extraction, these 40-h-old bacterial cultures were centrifuged at 3450 *g* for 5 min and the pellets were washed twice with Tris (10 mM, pH 8.3).

Protein extraction

Soluble proteins from *S. scabiei* cells grown with or without suberin were extracted using the phenol extraction method proposed by Hurkman and Tanaka (1986). Bacterial cells from a 30-mL culture were finely powdered in liquid nitrogen using a pestle and mortar. They were then resuspended in 8 mL of extraction

buffer [0.7 M sucrose, 0.5 M Tris, pH 7.5, 0.5 M EDTA, Complete (Roche Applied Sciences, Indianapolis, IN) 1 tablet/20 mL, 2% mercaptoethanol]. Cells were disrupted in a Fastprep 120 cell disrupter (Thermo Electron Corp., Milford, MA) for 45 s in the presence of 0.1-mm glass beads at speed setting 6.5. An equal volume of equilibrated phenol (Anachemia Canada Inc., Lachine, Canada) was added and the disruption procedure was repeated twice. The homogenate was centrifuged at 2500 *g* for 5 min and the proteins were extracted from the phenol phase with an equal volume of extraction buffer. Proteins were then precipitated with five volumes of cold 0.1 M ammonium acetate in methanol for a minimum of 30 min at -20°C and recovered from the suspension by centrifugation at 2500 *g* for 30 min. The protein pellet was finally resuspended in IEF buffer [9 M urea, 2% CHAPS, 1% Pharmalyte 3-10 (Amersham Biosciences, Uppsala, Sweden), 15 mM DTT] and the insoluble material was removed by centrifugation at 14 000 *g* for 15 min. Protein concentration was measured using the BioRad protein assay (Bio-Rad Laboratories, Hercules, CA) modified as follows. Twenty microlitres of a protein sample was mixed with 80 μL of 0.12 N HCl. Bio-Rad dye reagent was diluted 1:4 in water and filtered. Bio-Rad solution (3.5 mL) was added to each assay tube. The absorbance of the solution was measured at 595 nm after 5 min of incubation at room temperature. A standard curve, established with known concentrations of bovine serum albumin, was used to determine the protein concentration of the experimental samples.

Two-dimensional gel electrophoresis

For preliminary tests, 11-cm immobilized pH 4–7 gradient (IPG) strips (Amersham Biosciences) were used. For analytical purposes, 24-cm IPG strips covering pH ranges 4.5–5.5 and 5.5–6.7 were used. Strips were first rehydrated with between 300 μg and 1.8 mg of protein at 100 V for 12 h. Isoelectric focusing (IEF) was carried out using the IPGphor IEF System (Amersham Biosciences) for a total of 170 kV.h. Strips were then equilibrated for 15 min by incubating them with shaking in a solution comprising 0.05 M Tris pH 8.8, 2% SDS, 6 M urea, 30% glycerol containing 1% DTT and then for an additional 15 min in the same buffer in which DTT was replaced with 4% iodoacetamide. Strips were transferred onto ExcelGel SDS Homogeneous 15 (Amersham Biosciences) and SDS-PAGE was carried out according to the manufacturer's instructions. Broad-range protein markers (New England Biolabs, Ipswich, MA) were prepared according to the manufacturer's instructions on Whatman paper (Whatman Ltd, Maidstone, UK), and run alongside IEF strips. Proteins were stained with Coomassie brilliant blue R250 or were silver stained using a protocol compatible with mass spectrum analysis (Mortz *et al.*, 2001). Image analysis was carried out using Phoretix 2D software (Nonlinear dynamics, Newcastle upon Tyne, UK). Proteomes of cells grown with or without suberin were compared. Three experiments were

used to identify protein spots that were only associated with the presence of suberin.

N-terminal sequencing

In order to obtain the N-terminal protein sequence of some of the protein spots of the 2D gel, proteins were transferred to PVDF membranes by electrophoretic transfer (25 mM TRIS, 192 mM glycine, 20% methanol) and were visualized with Ponceau Red. The protein spots were excised from the membrane and used directly for automated Edman degradation on an Applied Biosystems model 473A pulsed-liquid protein sequencer (Foster City, CA). Amino acid sequences, obtained from automated Edman degradation, were used to search the completed genomes in the NCBI database (<http://www.ncbi.nlm.nih.gov>) using BLAST (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

Protein in-gel digestion and mass spectrometry

In order to obtain protein identification by mass spectrometry, bands of interest were excised from the 2D gels using a scalpel and placed in 96-well plates and then washed with water. The excised protein spots were digested with trypsin using a MassPrep liquid handling robot (Waters) according to the manufacturer's specifications and according to the protocol of Shevchenko *et al.* (1996) with the modifications suggested by Havlis *et al.* (2003). Briefly, the excised spots were destained with 50 μL of 50 mM ammonium bicarbonate and 50 μL acetonitrile, washed once with 50 μL of 100 mM ammonium bicarbonate and then dehydrated with 50 μL of acetonitrile. The in-gel proteins were reduced with 10 mM DTT for 30 min at 37°C and alkylated with 55 mM iodoacetamide for 30 min at room temperature. Trypsin digestion was performed using 105 mM of modified porcine trypsin (sequencing grade; Promega, Madison, WI) at 58°C for 1 h. The digested protein was extracted twice first with 30 μL of 1% formic acid/2% acetonitrile followed by 12 μL 1% formic acid/50% acetonitrile.

The recovered peptide extracts were pooled, vacuum centrifuge dried and then resuspended in 5 μL of 0.1% formic acid, and peptides were analysed for protein identification by mass spectrometry.

The digested protein samples were introduced into a tandem mass spectrometer, LCQ deca XP ion trap or LTQ linear ion trap (ThermoFisher) equipped with a Thermo Surveyor MS pump (ThermoFisher) using a PicoFrit column BioBasic C18, 10 cm $\times$ 0.075 mm internal diameter (New Objective, Woburn, MA) at a flow rate of 200 nL/min with a linear gradient from 2% acetonitrile in 0.1% formic acid to 50% acetonitrile in 0.1% formic acid over 30 min. MS/MS spectra were acquired by Xcalibur software version 2.0 (ThermoFisher) using a data-dependent acquisition mode. The MS survey scan was 400–2000 *m/z* and

was followed by collision-induced dissociation of the seven most intense ions for LTQ or the three most intense ions for the LCQ. The internal parameters was set to 30 s for dynamic exclusion and the relative collisional fragmentation energy was set to 35%.

Sequence analysis

All MS/MS spectra were analysed using Mascot (Matrix Science, London, UK; version 2.2.0) for peptide identification. Mascot was set up to search the bacteria Uniref100 database (version 8.0, 87 442 entries) assuming the digestion enzyme trypsin. The following search criteria were used: two missed cleavages, iodoacetamide derivative of cysteine was specified as a fixed modification and oxidation of methionine was specified as a variable modification. Peptide tolerance was 2.0 Da for the precursor and 0.5 Da for MS/MS. Score Mascot corresponded to $10 \times \log(P)$, where P is the probability that the observed match with a given MS/MS spectra is a random event. A high score indicates identity of extensive homology.

Each peptide sequence obtained by mass spectrometry (through the protein identification software Mascot) was compared by Blast analysis against the Sanger Institute *S. scabiei* genome sequence (protein versus translated DNA). The complete corresponding protein-coding gene was predicted using the GeneMarkHMM for Prokaryotes (Version 2.4) program. Entire deduced amino-acid sequences were compared by Blast analysis against the NCBI protein database for protein identification.

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