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ORIGINAL PAPER

Detection of a phage genome carrying a zonula occludens like toxin gene (*zot*) in clinical isolates of *Stenotrophomonas maltophilia*

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Abstract During a study of the genetic diversity of *Stenotrophomonas* strains, we found an autonomous replicating DNA molecule in chromosomal DNA preparations of the clinical *Stenotrophomonas maltophilia* strain c5. The entire sequence of 6,907 bp of the isolated DNA molecule was determined, which was called ϕSMA9. Seven ORFs, which code for proteins with considerable similarity to proteins in databases, were identified in the DNA sequence. The largest ORF shows high sequence similarities to the pI protein of the filamentous phage ϕLf, which was later shown to be identical to toxin Zot of *Vibrio cholerae*. Beside the Zot-like protein, six other proteins with similarities to known phage proteins such as a phage replication protein RstA and phage absorption or coat protein are encoded on ϕSMA9, which indicate that this circular DNA molecule represents the replicative form of a linear phage genome. A PCR-based screening showed that only five from the totally investigated 47 *Stenotrophomonas* strains of clinical and environmental origin harbor these genes. Altogether, we describe the first genome of a phage for the nosocomial pathogen *Stenotrophomonas*, which contains a Zot toxin like gene and might be regarded as the first *Stenotrophomonas* virulence factor.

Keywords Clinical isolates · Cluster analysis · Phage sequence

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Introduction

The genus *Stenotrophomonas*, a very heterogenous group within the γ-subclass of *Proteobacteria*, comprises five recognized species: *Stenotrophomonas acidaminiphila*, *S. maltophilia*, *S. nitritireducens*, *S. rhizophila*, and *S. koreensis* (Palleroni and Bradbury 1993; Berg et al. 1999; Finkmann et al. 2000; Wolf et al. 2002; Yang et al. 2006). *Stenotrophomonas* species play an important role in nature, especially in the global element cycle (Ikemoto et al. 1980), and they are frequently used in applied microbiology and biotechnology, e.g., in biological plant protection and biodegradation of xenobiotic compounds (Berg et al. 1994; Binks et al. 1995; Nakayama et al. 1999; Dunne et al. 2000). Furthermore, over the last decade multidrug-resistant *S. maltophilia* has become increasingly significant causing death in certain patient populations, particularly in those who are severely debilitated or immunosuppressed (for a review, see Denton and Kerr 1998). *S. maltophilia* is associated with a broad spectrum of clinical syndromes, e.g., bacteremia, endocarditis, respiratory tract infections etc. (Schaumann et al. 2001), and has an increasing importance for patients with cystic fibrosis (Steinkamp et al. 2005). *Stenotrophomonas* isolates occur ubiquitous and cosmopolitan, and were preferentially isolated from the

rhizospheres of diverse plants all over the world especially those of *Brassicaceae* (Berg et al. 1996). In addition, the bacterium has been isolated from a wide range of nosocomial sources, e.g., contact lens care systems, dialysis machines, ice-making machines, nebulizers and inhalation therapy equipment (Denton and Kerr 1998).

Although some traits have been implicated in the virulence of *Stenotrophomonas* using rat-lung infection (McKay et al. 2003) and *Dictyostelium discoideum* (Alonso et al. 2004) models of virulence, the basis of the pathogenicity of this bacterial genus is not well understood. *Stenotrophomonas* strains of environmental and clinical origin are able to adhere to many surfaces, e.g., to human epithelial respiratory cells (De Abreu Vidipó et al. 2001; De Oliviera-Garcia et al. 2003) and they are equally able to colonize the rhizosphere of strawberry (Suckstorff and Berg 2003). Furthermore, it was reported that many strains have the capability to produce extracellular enzymes, e.g., DNase, RNase, proteases, lipases, chitinases and elastases. Antibiotics synthesized by *Stenotrophomonas* are involved in pathogen suppression on plants whereas their importance for pathogenicity is still unclear (Jacobi et al. 1996; Nakayama et al. 1999). *S. maltophilia* strains are often highly resistant to multiple antibiotics (Alonso and Martinez 1997). Results obtained by Minkwitz and Berg (2001) indicated that the antibiotic resistance profile of *S. maltophilia* isolates was not associated with their origin (e.g., clinical and environmental especially from the rhizosphere). In addition, a method to differentiate between clinical and environmental *S. maltophilia* strains could not be established. The bivalent feature of *S. maltophilia*, highly useful for biotechnology and potential human-pathogenic make it necessary to investigate the bacterial diversity and pathogen related genes among diverse isolates of *Stenotrophomonas* with much more intensity.

Until now no toxin-related factor has been detected in any *Stenotrophomonas* strain, in contrast to human pathogen bacteria such as *E. coli* (hemolysin), *Vibrio cholerae* (cholera toxin) and others (Berg et al. 2005). In pathogenic strains of *V. cholerae* a second enterotoxin, the zonula occludenens toxin (Zot) was detected and found to be responsible for diarrheagenicity in strains defective of the cholera toxin (Fasano et al. 1991). Interestingly, a similar gene was found in the genome of the plant pathogen *Xanthomonas campestris*, which is known to induce lesions filled with water leaking from the surrounding plant tissue (Block et al. 2005). Here, we report the occurrence of a *zot*-like gene in *S. maltophilia* strains of clinical origin. Moreover, the gene is obviously located on a phage genome, which may be a vector to mobilize the toxin gene among *S. maltophilia* strains. This is the first identification of a presumable mobile toxin gene in strains of the nosocomial pathogen *S. maltophilia*, which should be screened in the future in *Stenotrophomonas* isolates in hospitals to evaluate its pathogenic potential.

Material and methods

Strains and growth conditions

All *Stenotrophomonas* strains (see Table 1) were cultivated in Luria-Bertani (LB) broth (LB broth base, ROTH, Kalrsruhe, Germany) or on LB agar plates. The clinical *Stenotrophomonas* strains and the *S. maltophilia* type strain DSM 50170 (ATCC 13637) were incubated at 37°C, while the environmental strains including the *S. rhizophila* type strain DSM 14405, isolated from the rhizosphere of oilseed rape (Wolf et al. 2002), were cultivated at 30°C. The majority of isolates (c1–20, e1–e20) had already been characterized pheno- and genotypically such as antibiotic resistance profiles and 16s rDNA sequences (Berg et al. 1996; Minkwitz and Berg 2001). Strains c1–c20 were isolated in the Rigshospitalet Copenhagen (Denmark) from various sites (tracheal aspirates, sputa, blood, throat, wounds, skin, ulcers, drainage fluids and aspirates, catheters, urine, etc. (Gerner-Smidt et al. 1995). Strains c21–c25 originally isolated from humans were obtained from a reference laboratory in Leipzig (Germany).

DNA isolation

Total DNA from the *Stenotrophomonas* strains was extracted after lysozyme treatment and purified by the CTAB method as described elsewhere (Hagemann et al. 1997). Plasmid isolation was carried out using the Qiagen® Plasmid Midi Kit (Qiagen, Hilden, Germany) according to the manufactures manual for low copy plasmids. Bacterial DNA for PCR analyses was prepared by boiling a bacterial colony for 5 min in water.

Southern-blot analysis

Approximately 5 μg of total DNA obtained by the CTAB method were cut with 10 units of *Pst*I or *Bcl*I at 37°C for 16 h. DNA fragments were separated on a 0.8% agarose gel and transferred to a nylon membrane (Roti-Nylon plus, ROTH, Karlsruhe, Germany) by semi-dry blotting. DNA fragments of the coding

Table 1 List of all investigated strains of *S. maltophilia* (*S.m.*) and *S. rhizophila* (*S.r.*) which were isolated from clinical (c) and environmental (e) sources (Berg et al. 1999), respectively, regarding the occurrence of a PCR product of the *zot* or *rstA* genes ('+' DNA fragment present and '–' DNA fragment absent)

Number	Isolate no.	Species	*zot*	*rstA*
1	C1	*S.m.*	–	–
2	C2	*S.m.*	–	–
3	C3	*S.m.*	–	–
4	c4	*S.m.*	+	+
5	c5	*S.m.*	+	+
6	c6	*S.m.*	+	+
7	c7	*S.m.*	+	+
8	c8	*S.m.*	–	+
9	c9	*S.m.*	–	–
10	c10	*S.m.*	+	+
11	c11	*S.m.*	–	–
12	c12	*S.m.*	–	–
13	c13	*S.m.*	–	–
14	c14	*S.m.*	–	–
15	c15	*S.m.*	–	–
16	c16	*S.m.*	–	–
17	c17	*S.m.*	–	–
18	c18	*S.m.*	–	–
19	c19	*S.m.*	–	–
20	c20	*S.m.*	–	–
21	c21	*S.m.*	–	–
22	c22	*S.m.*	–	–
23	c23	*S.m.*	–	–
24	c24	*S.m.*	–	–
25	c25	*S.m.*	–	–
26	c26	*S.m.* (DSM 50170)	–	–
27	e-a1	*S.m.*	–	–
28	e-a2	*S.m.*	–	–
29	e-p12	*S.m.*	–	–
30	e-p13	*S.m.*	–	–
31	e-a21	*S.m.*	–	–
32	e-a22	*S.m.*	–	–
33	e-a23	*S.m.*	–	–
34	e-a24	*S.m.*	–	–
35	e-p3	*S.m.*	–	–
36	e-p4	*S.m.*	–	–
37	e-p5	*S.m.*	–	–
38	e-p8	*S.m.*	–	–
39	e-p6	*S.r.*	–	–
40	e-p7	*S.r.*	–	–
41	e-p9	*S.r.*	–	–
42	e-p10	*S.r.* (DSM 14405)	–	–
43	e-p11	*S.r.*	–	–
44	e-p14	*S.r.*	–	–
45	e-p15	*S.r.*	–	–
46	e-p16	*S.r.*	–	–
47	e-p17	*S.r.*	–	–

sequences of *zot* or *rstA* were used as probes in Southern blotting experiments. For amplification of the gene-specific probes diluted DNA of the isolated phage replicon ϕSMA9 served as a template. Gene-specific primers were newly generated using our complete phage sequence (*zot*: 5′-GTT GCT CAC ACT GGT GG-3′ and 5′-GAG CGT CGG ATC TTC CT-3′; *rstA*: 5′-GGT CAG CAG GAC GCA AT-3′ and 5′-CCT TGA CGG CTA AGC CA-3′). The gene fragments were amplified and labeled by digoxigenin using the PCR DIG Probe Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The blotted DNA was incubated with individual labeled DNA-probes for 16 h at 42°C in a formamide containing hybridization solution (Hagemann et al. 1997). After first wash with 2× SSC buffer containing 0.1% SDS at 68°C for 45 min and a second wash with 0.1× SSC buffer containing 0.1% SDS at room temperature the bound Dig-labeled probes were detected using the DIG DNA and Detection Kit (Roche, Germany).

Molecular typing by BOX-PCR

Genomic DNA from each strain was extracted by the method of Anderson and McKay (1983). BOX element oligonucleotide primers with the sequence of 5′-CTACGGCAAGGCGACGCTGACG-3′ were synthesized by MWG Biotech (Ebersberg, Germany). The PCR reactions were performed as previously described by Rademaker and de Bruijn (1992) in duplicate for each isolate. Computer assisted evaluation of the BOX-PCR-generated fingerprints was made using the GelCompare program (version 4.1; Applied Math, Kortrijk, Belgium).

Restriction- and sequence analyses

First characterization of ϕSMA9 was done by restriction analyses. The restriction enzymes were purchased from NEB (New England Biolabs). The DNA sequencing after shot-gun cloning of *Sau*3A-fragments has been carried out by the company Seqlab (Sequence Laboratories, Göttingen, Germany). The entire sequence was determined with threefold–tenfold coverage and was submitted to the EBI database (accession number AM040673). Potential protein coding regions (ORFs) were searched on the sequenced DNA. At all about 20 ORFs were predicted, which overlapped in some cases. The deduced amino acid sequences of all potential ORFs were compared to databases using the software ORF-Finder and Blast (Altschul et al. 1997) at NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). In parallel overlapping, 500 bp pieces of the DNA (BlastX) were used for comparisons.

Results

During the study of the genetic diversity among isolates from clinical and environmental sources, which

belong to the species *S. maltophilia* or *S. rhizophila* (see Table 1), we found indications for the occurrence of an autonomous replicating DNA molecule in chromosomal DNA preparations from the clinical *S. maltophilia* strain c5 (Fig. 1a). Using a plasmid purification protocol, the extra-chromosomal DNA molecule was isolated. The isolated DNA ran in two bands (major band at around 8 kb and a weaker one higher than 22 kb) in an agarose gel (Fig. 1b), which is characteristic for circular DNA such as plasmids. After restriction analysis, we found that the enzymes *Pst*I and *Apa*I seem to cut ones inside the DNA molecule leading to one fragment of about 7 kb (Fig. 1b). Treatment with other enzymes resulted in several fragments, the sizes of these fragments summed up again to approximately 7 kb. The restriction pattern indicated that *S. maltophilia* strain c5 harbored an extra-chromosomal, circular DNA-molecule of about 7 kb, which could represent a plasmid or small phage genome.

The sequence of the isolated DNA molecule was estimated by shot-gun sequencing after cloning of 96 fragments generated by partial *Sau*3A digestion. The entire sequence of 6,907 bp was covered at least three times by single sequences of the clone library, for about 90% of the sequence tenfold coverage was obtained. The sequencing verified the circular nature of the isolated DNA fragment, since three different fragments overlapped the artificial 5′ and 3′ end of the estimated sequence file. After circularization, the DNA was cut in silico by the enzymes used before in restriction analysis. The experimental observed fragment pattern was exactly reproduced (cf. Figs. 1b, 2). Seven ORFs were identified, which code for proteins with considerable similarities to proteins in databases (Fig. 2, Table 2). All other potential ORFs were excluded here and will not be discussed in the present paper, since its protein coding capacity is very speculative. The entire DNA sequence is deposited in EBI database under the accession number AM040673 and in GenBank under the accession number NC 007189.

The analysis of the complete genome sequence resulted in two main findings. First, the sequenced circular DNA molecule seems to represent rather the replicative form of a linear phage genome than a plasmid. The newly discovered phage was named ϕSMA9, since eight different phages designated ϕSMA1–8 were recently described for *S. maltophilia* strains of different origin (Chang et al. 2005). The size and gene organization of the ϕSMA9 genome was quite similar to genomes of the phages ϕLF from *X. campestris*, Xfϕf1 from *Xylella fastidiosa* and CTXϕ from *V. cholerae* (http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/29258.html). Second, the largest protein coding region of ϕSMA9 can be translated into a protein of 442 amino acid residues showing considerable high sequence identity to the pI protein of the filamentous phage ϕLf which was later shown to be identical to Zonula occludens toxin (Zot) (Fasano et al. 1991). The putative Zot represents the first toxin identified in a strain of the nosocomial pathogen *S. maltophilia*. A Zot protein of high similarity was found on the chromosome of *Xylella fastidiosa* (48% identity) and a protein with lower similarity is encoded in the pathogen island of *Vibrio cholerea* (24% identity) (Fig. 3 and Table 2). The similarity with Zot can be observed over the entire protein sequence. Particularly, the N-terminal part is well conserved. Interestingly, a close homolog to Zot from ϕSMA9 was also identified in the complete sequence of the related plant pathogen *X. campestris* (Table 2), while it is absent from the unfinished genome sequence of *S. maltophilia* strain K279a (http://www.sanger.ac.uk/Projects/S_maltophilia/). These significant sequence similarities indicate that on ϕSMA9 indeed a Zot protein is encoded.

Beside Zot, six other proteins with similarities to known phage proteins are encoded on ϕSMA9. The occurrence of typical phage genes clearly indicates that this circular DNA molecule is not a plasmid but an autonomous phage replicon. Particularly, the second largest protein on ϕSMA9 shows significant identities

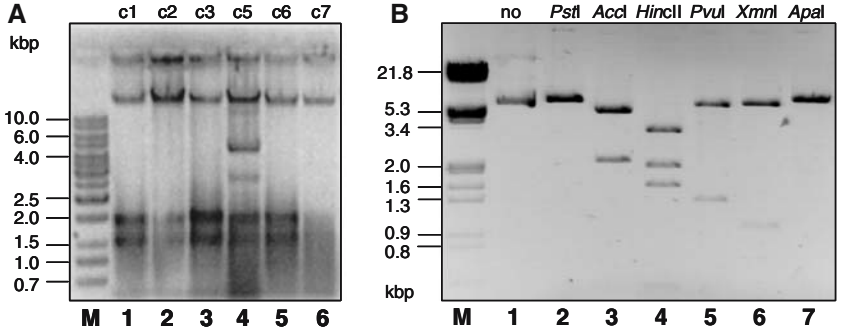

Fig. 1 Detection and characterization of the extra-chromosomal DNA molecule in *S. maltophilia* strain c5. **a** Separation of total DNA from selected *S. maltophilia* strains using agarose (0.8%) gel electrophoresis. **b** Restriction analysis of the isolated extra-chromosomal DNA molecule from *S. maltophilia* strain c5

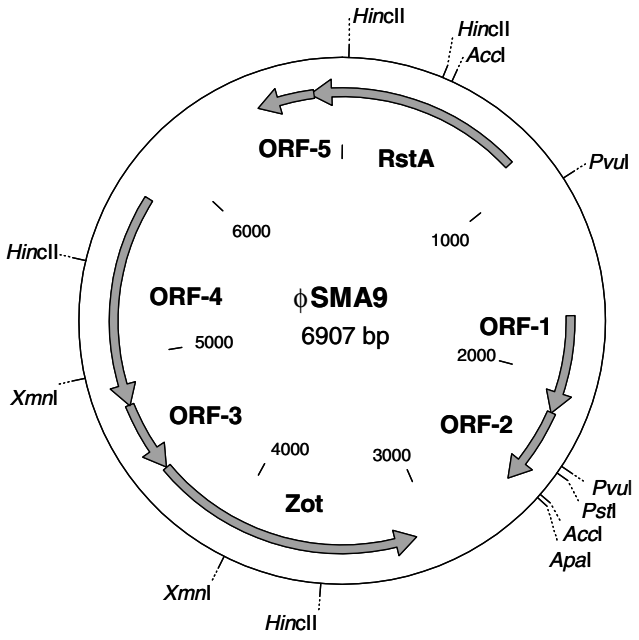

Fig. 2 Physical map and identified ORFs on the genome of phage ϕSMA9 from *S. maltophilia* strain c5. The DNA was completely sequenced after shot-gun cloning. The obtained DNA was circularized and ORFs were identified using the software packages BlastX, BlastP and ORF finder. Only ORFs coding for proteins showing significant similarities to proteins in databases are shown (see Table 2). Selected restriction sites are indicated, which were used to characterize the isolated DNA of ϕSMA9 (see Fig. 1b)

to the so-called phage replication proteins, RstA (Table 2). Again, the high degree of sequence similarity can be seen over the whole protein, which shows 46% identities with phage replication proteins from filamentous phages such as ϕLf or ϕXo isolated before from members of the closely related genus *Xanthomonas*. Moreover, in such phages the *zot* gene was first described. Interestingly, again a close homolog to RstA on ϕSMA9 was identified in the genome of *X. campestris* (Table 2). Beside the phage replication protein, other phage-related proteins possibly involved in phage absorption or coat protein synthesis were found among the ORFs 1–5 of ϕSMA9, which occur also in the genomes of *X. fastidiosa* or *X. campestris* (Table 2).

Further PCR screenings were performed to check the distribution of the phage genes *zot* and *rstA* among different strains of *S. maltophilia* and *S. rhizophila*. Only in a few *S. maltophilia* strains of clinical origin these genes are present, since only with DNA from some clinical strains (c4, c5, c7, and c10) the expected fragments were obtained (Table 1), while the majority of clinical isolates and the environmental isolates of *S. maltophilia* as well as *S. rhizophila* did not harbor these genes (not shown). In order to verify this observation, the PCR positive strains and some additional strains were screened by Southern-blot analysis using gene probes specific for *zot* or the phage-replication protein *rstA*. Again, specific signals were only obtained with DNA from a few strains of clinical origin (Fig. 4). The type strains for the species *S. maltophilia* and *S. rhizophila* did not contain any DNA homologous to the applied DNA probes. Despite the same amount of DNA was loaded on the gel, the most intensive signal was obtained with total DNA from strain c5 (Fig. 4a), the original source for the isolation of ϕSMA9. Moreover, the *zot* gene could be also detected in DNA of strains *S. maltophilia* c4 and c10, respectively, which showed similar fragment patterns

Table 2 Protein-coding regions identified on the sequenced circular phage replicon in *S. maltophilia* strain c5 (see Fig. 2, AM040673)

ORF no.	Start (bp)	Stop (bp)	AS res.	Putative protein function	GI no.	e Value
Zot	4,416	3,088	442	pI protein (*X. campestris* pv. *campestris* ATCC 33913)	21231516	$1e^{-128}$
				pI protein—filamentous phage ϕLf	7520811	$1e^{-128}$
				Zonula occludens toxin (*Xylella fastidiosa* Dixon)	53801172	$1e^{-110}$
RstA	901	6,753	351	Phage replication protein RstA (*Xylella fastidiosa* Ann-1)	52856586	$2e^{-76}$
				Phage replication initiation factor (*Acinetobacter* sp. ADP1)	50085003	$3e^{-24}$
1	1,702	2,184	160	Phage-related protein XF1864 (*Xylella fastidiosa* 9a5c)	9106953	$9e^{-6}$
				Phage-related protein (*X. campestris* pv. *campestris* ATCC 33913)	21231505	$2e^{-4}$
2	2,181	2,561	126	ORF112 (*X. campestris* phage ϕLf)	311542	$6e^{-16}$
				Phage-related protein (*X. campestris* pv. *campestris* ATCC 33913)	21231514	$2e^{-13}$
3	4,765	4,418	115	Minor coat protein (*X. campestris* pv. *campestris* ATCC 33913)	21231517	$7e^{-23}$
				Phage-related protein (*Xylella fastidiosa* Temecula1)	28198836	$3e^{-19}$
4	5,799	4,762	345	Phage-related protein (*Xylella fastidiosa* Temecula1)	28198837	$6e^{-11}$
				Adsorption protein pIII (*Xanthomonas oryzae* phage ϕXo)	15054486	$1e^{-8}$
5	6,771	6,493	92	Phage-related protein (*Xylella fastidiosa* Temecula1)	28198840	$8e^{-10}$
				Single-stranded DNA binding protein (*X. campestris* pv. *campestris* ATCC 33913)	21231522	0.003

Only protein-coding regions are listed, which show significant sequence similarities to entries in databases

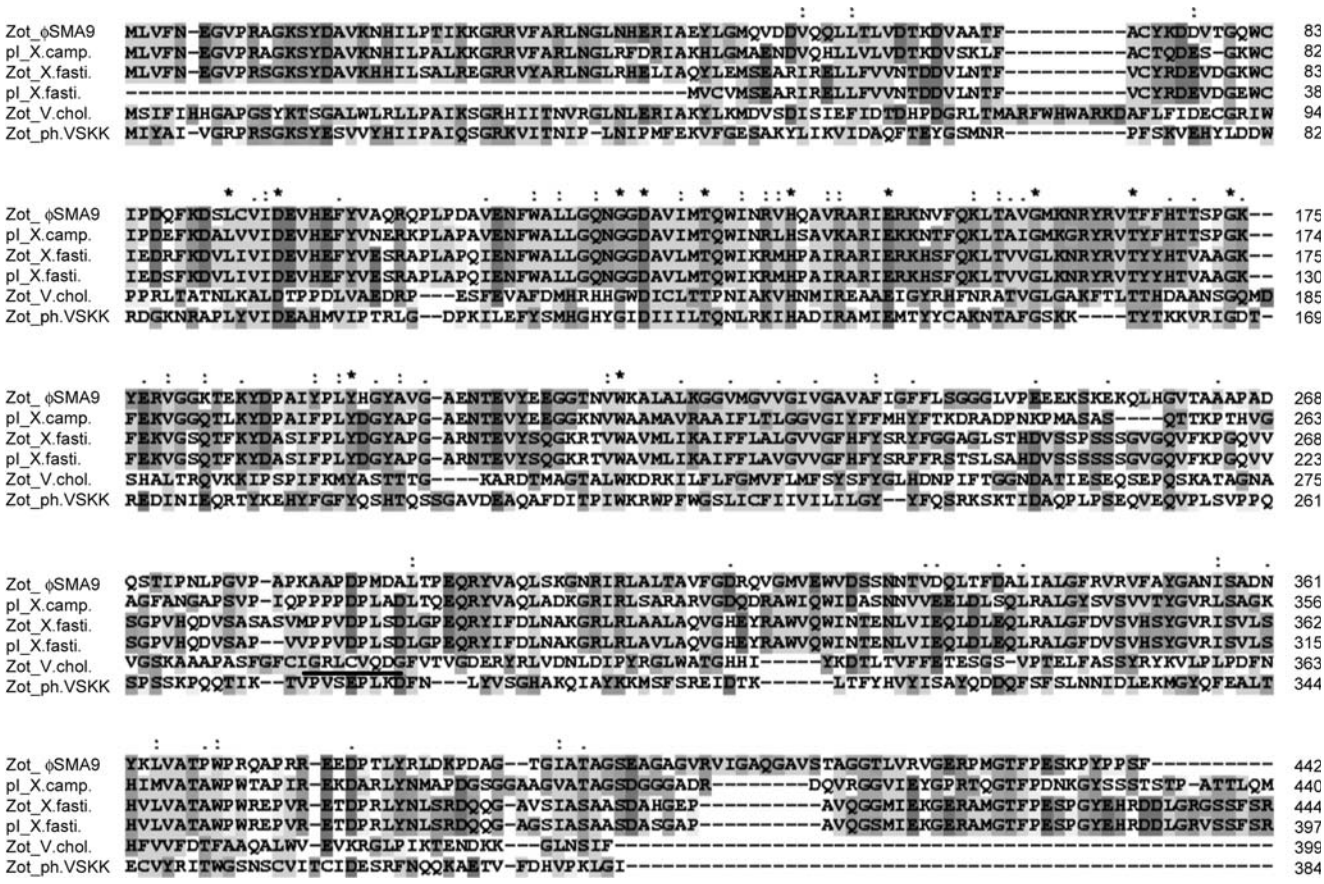

Fig. 3 Amino acid sequence comparison of the putative Zot (*Zot_ϕ*SMA9 putative zonula occludens toxin) from *ϕ*SMA9 of *S. maltophilia* strain c5 with similar proteins from databases using ClustalX. *pI_X.camp.* pI-protein from *X. campestris*, gi|21231516; *Zot_X.fasti.* Zot from *Xylella fastidiosa*, gi|53801172; *pI_X.fasti.* phage-related protein, *Xylella fastidiosa*, gi|28198835; *Zot_V.chol.* Zot from *V. cholerae*, gi|282084; *Zot_ph.VSKK* Zot from phage VSKK, gi|17530552; *asterisk* identical amino acid residues in all sequences, *vertical double dot* similar amino acid residues in all sequences; *single dot* similar amino acid residues in most sequences, *underlined* putative zonulin receptor binding sequence in Zot from *V. cholerae* according to Di Pierro et al. (2001)

as DNA from c5 or the isolated DNA *ϕ*SMA9 (Fig. 4a). Weaker but significant signals with different fragment sizes were also obtained with DNA from strain c7 for the *zot* gene. Interestingly, the phage replication protein gene was absent from strain c7 in Southern-blotting (Fig. 4b) as well as PCR experiments, while it was detectable in the strains c5, c4 and c10. Again the fragment labeling is much weaker in the strains c4 and c10, and, furthermore, their fragment sizes are different from the strain c5 and the isolated DNA of *ϕ*SMA9 (Fig. 4b). Obviously, the *zot* gene harboring phage is restricted to a few clinical isolates and seems to be differentially organized in the different strains.

In a last attempt, all strains investigated for the occurrence of *zot* or *rstA* were compared using the molecular fingerprint method BOX-PCR (Fig. 5). The cluster analysis revealed many sub-clusters among the *S. maltophilia* strains, most of them contain only clinical isolates. Interestingly, one sub-cluster showing less than 50% similarity in the BOX-PCR fragment pattern compared to other *S. maltophilia* clusters contained all the strains with positive signals for *zot* and *rstA*. The three strains c4, c5 and c10 showing the strongest signals cluster closely together, while the strain c7, which has only *zot* but not the phage replication protein gene, grouped slightly distant with two other clinical isolates (Fig. 5). A separate branch for the strains c4, c5 and c10 was also observed in the 16S rDNA sequence-based cluster analysis of *S. maltophilia* isolates (not shown).

Discussion

The present study describes the first phage genome from the nosocomial pathogen *S. maltophilia*. Before, a small plasmid pNKH43 (accession number L09673,

Henry et al. unpublished) has been identified in this bacterium; related plasmids were reported from *Xylella* and *Xanthomonas*. Blast searches (Altschul et al. 1997) using the DNA and/or protein sequences did not show any similarity of the pNKH43 to ϕSMA9. Recently, eight phages were isolated from *S. maltophilia* (Chang et al. 2005) but yet not sequenced. However, these phages were able to lyse the *S. maltophilia* host cells. The phage identified here seems to belong to the group of linear phages, which are exported from the host without its lysis. Accordingly, we never observed plaques in bacterial lawns of *S. maltophilia* strain c5. It was named ϕSMA9 following the nomenclature of Chang et al. (2005). After infection, a copy of the phage genome is incorporated into the host genome and is propagated as a prophage. In order to replicate, a double-stranded, plasmid-like virion molecule is made, which is also used to produce phage proteins and new phage particles. Such a replicative form of the filamentous phage ϕSMA9 has been obviously isolated from *S. maltophilia* strain c5. As described before for genomes of related phages, genes involved in phage export and chromosomal integration are absent from ϕSMA9. It has been shown for the related phages CTXϕ and ϕLF that site-specific recombination into the host occurs via specific integration sequences using host-encoded recombination enzymes (Huber and Waldor 2002; Lin et al. 2001). Moreover, it is known that the host specificity of such phages depends on the so-called phage absorption protein (Heipern and Waldor 2003; Lin et al. 1999). A homologous protein is encoded on the genome of ϕSMA9. Since the whole phage genome is similar to phages infecting closely related bacteria such as *Xanthomonas* and *Xylella*, one may speculate that subtle changes in those phage proteins made the infection of *S. maltophilia* possible.

The genome of ϕSMA9 harbored a putative *zot* toxin gene. This is the first possible virulence factor known from clinical isolates of *S. maltophilia*. The putative Zot of ϕSMA9 showed significant similarities to Zot from similar phages found in the bacteria *Xanthomonas* and *Xylella*. Furthermore, very similar gene copies were detected on the chromosome of these bacteria. To a lesser extent similarities to Zot from *V. cholerae* were detectable. The *Vibrio* Zot was identified as virulence factor affecting intestinal tissue

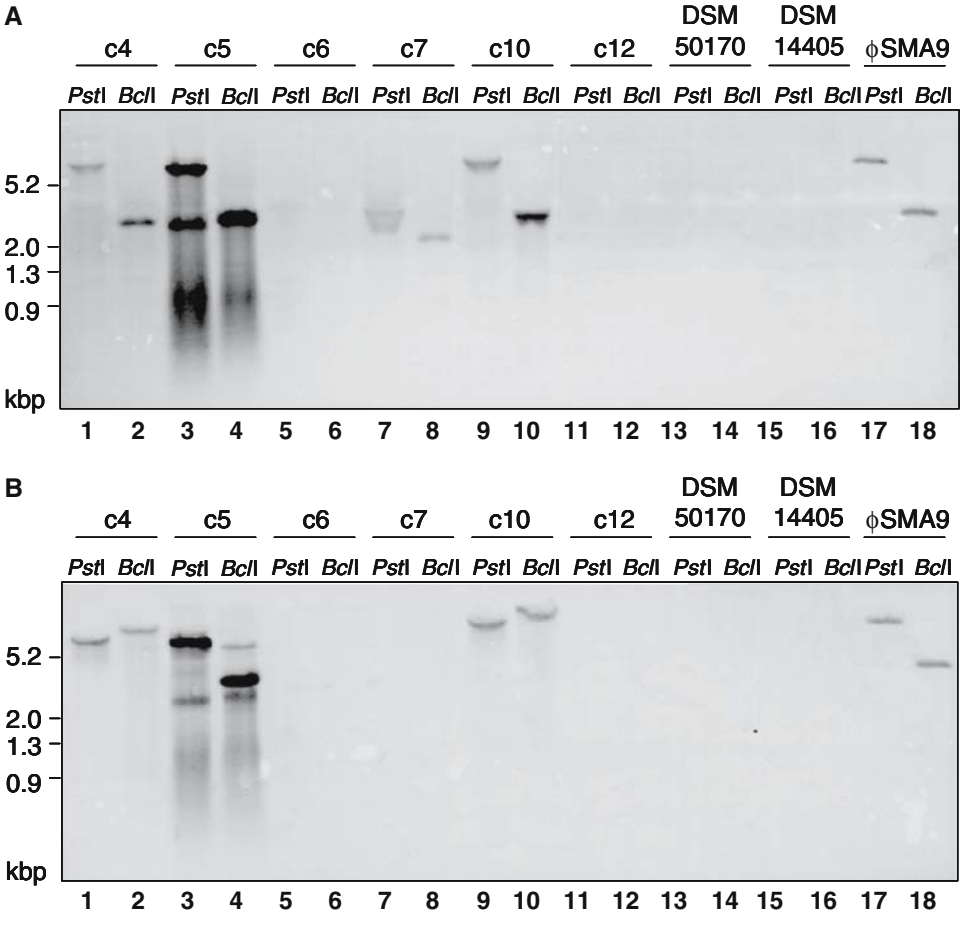

Fig. 4 Detection of *zot* (**a**) and *rstA* for phage replication protein (**b**) genes in different strains of *S. maltophilia* and *S. rhizophilia* by Southern-blot hybridization. Internal fragments of the *zot* (**a**) and *rstA* (**b**) from ϕSMA9 were labeled with digoxigenin and hybridized with chromosomal DNA from different clinical isolates of *S. maltophilia* (strains c4, c5, c6, c7, c10, and c12), the type strains of *S. maltophilia* (DSM 50170) and *S. rhizophila* (DSM 14405) or the isolated extra-chromosomal DNA molecule of phage ϕSMA9 from *S. maltophilia* strain c5 cut with *Pst*I (*odd numbered*) and *Bcl*I (*even numbered*), respectively

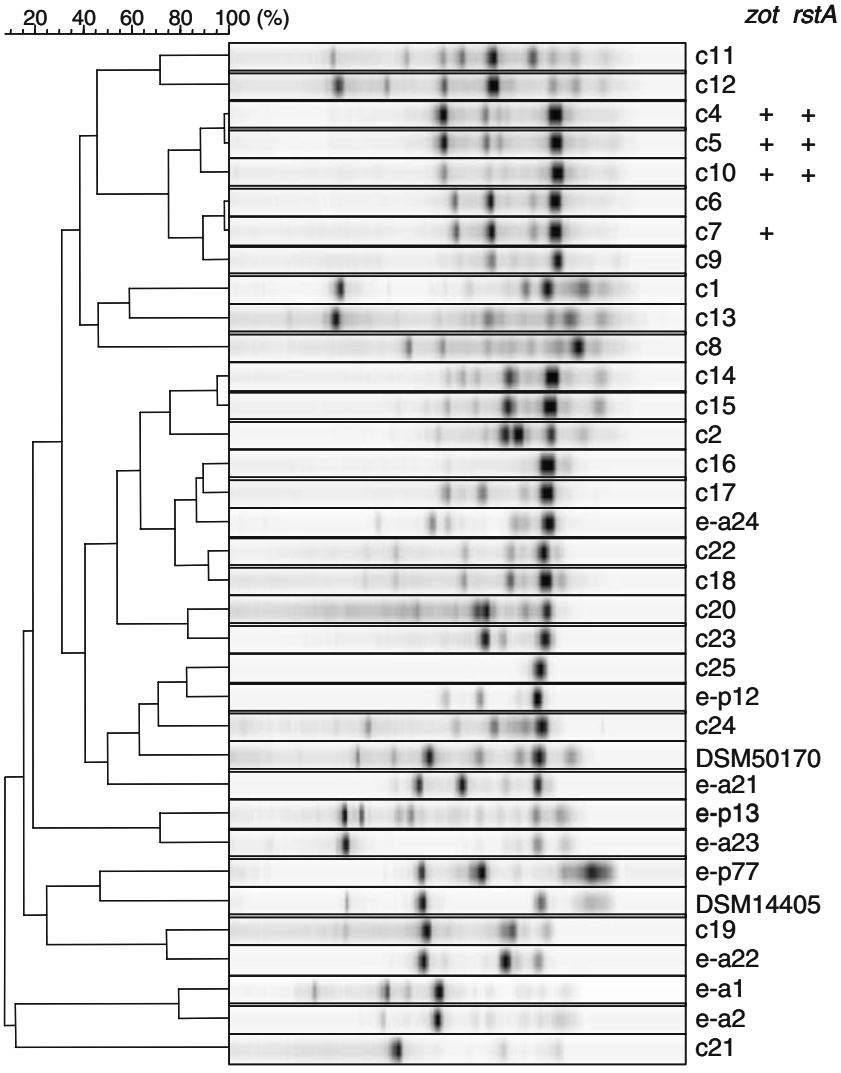

Fig. 5 Cluster analysis according to the fragment pattern obtained by the molecular fingerprinting method BOX-PCR with DNA from isolates of clinical (indicated by the letter 'c') or of environmental (indicated by the letter 'e') origin of the species *S. maltophilia* (type strain: DSM 50170) and *S. rhizophila* (type strain: DSM 14405), respectively. All strains were investigated regarding the occurrence of the *zot* or phage replication protein (*rstA*) gene ('+' and '–' genes detected by Southern-blot hybridization and PCR, respectively). Further descriptions of the strains are included in Berg et al. (1999)

additional to classical cholerae toxin CTX (Fasano et al. 1991). It was shown that binding of the *Vibrio* Zot modulated tight junction in intestinal cell cultures (Fasano et al. 1995). A detailed structure function study revealed that an extracellular, probably C-terminal proteolysis product of this toxin binds to a specific receptor on intestine epithelia cell envelopes. Binding to the receptor influences the membrane permeability resulting in considerable water loss from toxin affected cells (Di Pierro et al. 2001). The receptor binding site identified in Zot from *V. cholerae*, however, is not at all conserved in the *Stenotrophomonas* Zot protein. Moreover, the whole C-terminal part of Zot from ϕSMA9 is rather divergent from the *Vibrio* Zot (see Fig. 3). These sequence divergence make the toxic potential of the *Stenotrophomonas* Zot questionable. Probable, it represents rather a pI protein orthologue, which is found in all genomes of related filamentous phages. Those pI proteins are required for phage assembly (Chang et al. 1998).

PCR and Southern blot experiments revealed that the genes for Zot and phage replication protein RstA are restricted to a few clinical isolates of *S. maltophilia*. A complete correlation between both detection methods was observed, however, Southern blotting resulted in more reliable gene detection, since small sequence diversities may disturb primer binding in PCR analyses but is tolerated by the hybridization method. Both genes were not found in the majority of *S. maltophilia* strains including the type strain and none of the investigated *S. rhizophila* strains contained those genes. The same was found for the *zot* gene in *V. cholerae*, which was only found in specific isolates and was completely absent from many newly isolated strains in Brazil (Rivera et al. 2001). The Southern-blot analysis indicated diversity in the *zot* and *rstA*

sequences and occurrence. Particularly interesting is strain c7, because in this strain only *zot* but no *rstA* was found, while in strain c5 the fragment pattern indicates beside the autonomous copy a second copy of these genes on the chromosome. In the genome sequence of *X. campestris*, the phage genes were also found integrated on the chromosome and a *zot* gene at another site. Those prophages are typically made during the life cycle of linar phages.

Form these results, one can draw a hypothetical scenario that *zot* was acquired by the few clinical *Stenotrophomonas* strains only recently after infection by a filamentous phage, which probably came after certain changes in its adsorption protein from plant-pathogens such as *Xanthomonas* or *Xylella* spp.. All strains with *zot* and *rstA* cluster closely together indicating their origin from a common parental strain. Most probably, the strain c7 has already lost most of the phage genome. The phage insertion resulted in a mobile genetic element which is now able to transfer the toxin gene among *Stenotrophomonas* strains and possible enhance its pathogenic strength. In the case of *V. cholerae*, it is well established that filamentous phages are potent carriers for virulence factors (Davis and Waldor 2003). Therefore, the occurrence of ϕSMA9 and *zot* should be monitored in the future in clinical strains of *S. maltophilia*.

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