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Identification of the Exported Proteins of the Oral Opportunistic Pathogen *Actinobacillus actinomycetemcomitans* by Using Alkaline Phosphatase Fusions

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A *phoA* fusion library of *Actinobacillus actinomycetemcomitans* genomic DNA has been screened to identify genes encoding exported and secreted proteins. A total of 8,000 colonies were screened, and 80 positive colonies were detected. From these, 48 genes were identified with (i) more than half having homology to known or hypothetical *Haemophilus influenzae* genes, (ii) 14 having no ascribed function, and (iii) 4 having very limited or no homology to known genes. The proteins encoded by these genes may, by virtue of their presence on the cell surface, be novel virulence determinants.

Actinobacillus actinomycetemcomitans is implicated as a pathogen in one of the most prevalent diseases of humans—periodontitis (9). However, surprisingly little is known about the virulence factors of this organism (16). It is well recognized that bacterial exported proteins play key roles in many bacterial functions and are particularly important in the processes of infection (3). In order to gain some idea of the nature of the genes encoding exported proteins in *A. actinomycetemcomitans* use has been made of a plasmid-based *phoA* gene fusion system initially developed for studying protein secretion in *Escherichia coli* (5). This methodology has been used to identify and characterize exported proteins in a number of gram-negative and gram-positive bacteria (1, 4, 7, 10, 12), including a recent report of exported proteins of *A. actinomycetemcomitans* (8).

Bacterial strains and media. *A. actinomycetemcomitans* NCTC 9710 was grown on brain heart infusion agar (Oxoid) supplemented with 5% (vol/vol) horse blood in a carbon dioxide-rich atmosphere for 48 h. *E. coli* JM107 was grown on nutrient agar (Oxoid). *E. coli* CC118 was grown on nutrient agar containing erythromycin (ERY) (150 μg/ml).

Isolation of cell-associated proteins. A fraction containing the cell surface-associated proteins of *A. actinomycetemcomitans* was prepared by gentle saline extraction as described in reference (6).

Production of rabbit antisera to *A. actinomycetemcomitans* cell surface-associated proteins. Three rabbits were immunized with this saline wash of *A. actinomycetemcomitans* in a nonulcerogenic adjuvant and boosted with material in Freund's incomplete adjuvant. Animals were bled, and titers of antisera were assessed by enzyme-linked immunosorbent assay at various intervals, until titers peaked. Rabbits were then exsanguinated and sera were prepared using conventional means. The rabbit antisera were pooled and extensively immunoadsorbed with *E. coli* until binding to this bacterium was extinguished. Animal experimentation was done under United Kingdom Home Office regulations.

Construction of *phoA* fusion library. Chromosomal DNA was prepared from *A. actinomycetemcomitans* using standard methods (described in reference 13). Plasmid libraries containing DNA from *A. actinomycetemcomitans* were prepared in the plasmid vector pHRM104 (23). Chromosomal DNA was partially digested with *Sau*3AI for 3 h at 37°C. The vector DNA was extracted from 200 ml of overnight cultures of *E. coli* JM107 using a Midi plasmid preparation kit (Qiagen Ltd, Crawley, United Kingdom). The plasmid was linearized by digestion with *Bam*H1 for 2 h at 37°C. The partially digested chromosomal DNA was ligated with the linearized pHRM104 overnight in a ligation mixture consisting of 40 μl (5 μg) of *Sau*3AI fragments, 20 μl (1 μg) of linearized plasmid, 6 μl of ligase buffer, and 2 μl of T4 DNA ligase. To confirm that digestion and ligation had taken place, aliquots were removed from the various reactions and analyzed by agarose gel electrophoresis.

Transformation of *E. coli* CC118. Competent *E. coli* CC118 cells were prepared and transformed with the ligated DNA. The transformed cells were transferred into 5 ml of nutrient broth and incubated at 37°C with shaking for only 1.5 h. Cells were plated onto nutrient agar containing ERY (150 μg/ml) and 5-bromo-chloro-3-indoyl phosphate (XP) (40 μg/ml) a substrate for alkaline phosphatase, and incubated overnight at 37°C. Alkaline phosphatase-positive colonies were picked and subcultured onto fresh ERY-XP plates, and the presence of alkaline phosphatase activity confirmed. Plasmid DNA was extracted from individual alkaline phosphatase positive colonies and prepared using the Qiaprep spin miniprep kit. The sizes of the inserts in the recombinant plasmids were determined by digesting the DNA with *Kpn*I and running the digests on agarose gels.

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TABLE 1. *A. actinomycetemcomitans* clones showing homology to known *Haemophilus influenzae* proteins[a]

Clone[e]	No. of fused aa	ORF (full length)	Protein identity	% Similarity	% Identity	SP or TM[b]
1	43	1242 (356)	Peptide methionine sulfoxide reductase	85	73	SP
4	132	892 (476)	Murein peptide ligase	87	78	TM
11	191	2224 (461)	Anaerobic dimethylsulfoxide reductase (DmsA)	96	92	TM
14	388	306 (769)	Thiol:disulfide interchange protein (DsbD)	66	54	TM
15	373	(404 in HI)	Aminotransferase[c]	95	88	TM[3]
19	29	1171 (259)	Amino acid ABC transporter periplasmic component	66	49	SP
22	122	788 (356)	Type B outer membrane protein	69	58	SP
27	47	(162)	Hypothetical lipoprotein[2]	83	70	SP
28	47	604 (626)	Signal peptidase	75	60	SP
30	110	1365 (311)	Malate dehydrogenase	93	89	SP
31	152	(738)	Soluble lytic transglycosylase[2]	69	59	SP
35	85	1082 (514)	Oligopeptide transporter periplasmic binding protein (putative)	65	50	SP
37	58	2003 (423)	UDP-*N*-acetylglucosamine-1-carboxy vinyl transferase	92	87	TM
38	23	498 (809)	Recombination protein rec 2	67	52	TM
41	120	1715 (332)	D-XyloseABC transporter/periplasmic binding protein	92	85	SP
46	32	346 (127)	Thiol:disulfide interchange protein (*DsbE*)	83	72	SP
47	66	802 (810)	Probable organic solvent tolerance protein precursor	81	67	TM
56	37	1619 (470)	Aspartate aminotransferase	91	84	No
59	59	853 (396)	Bicyclomycin resistance protein homologue	85	72	SP
69	71	403 (591)	Penicillin binding protein 3	84	72	SP
72	51	778 (172)	Cytochrome *c* biogenesis protein	80	72	SP
73	77	1290 (313)	Cell division protein FTSH homologue 1	92	85	SP
79	170	1442 (484)	Cytochrome c_{552} formate-dependent nitrite reductase	89	80	SP
80	234	(487 in HI)	Colicin tolerance protein[2]	86	72	SP

[a] The table provides information on the number of amino acids in the protein fused to the PhoA, the ORF number (derived from the PEDANT database), the number of amino acids encoded by the full-length ORF, the identity of the protein, the identity and similarity to the same protein in the *H. influenzae* genome database, and whether the cloned protein has a signal peptide (SP) or transmembrane spanning (TM) segment.

[b] SP denotes the presence of a signal sequence within the N-terminal 50 to 70 amino acids using the program SignalP V1.1 (http://www.expasy.ch/tools/). TM denotes the presence of a transmembrane spanning segment using the programs DAS, HMMTOP, TMHMM, Tmpred, and TopPred 2 at http://www.expasy.ch/tools/.

[c] These DNA sequences are not yet in the PEDANT annotated *A. actinomycetemcomitans* database and thus do not have an ORF number.

[d] A weak transmembrane spanning segment is seen via the DAS programme in the segment fused to *phoA*.

[e] Numbers in boldface type represent clones that were recognized by the rabbit antiserum to a saline wash of *A. actinomycetemcomitans*.

Sequencing of alkaline phosphatase-positive clones. An oligonucleotide (5′-CGGTTTTCCAGAACAGG-3′) specific to the 5′ end of the truncated *phoA* gene in pHRM104 was used to sequence over the fusion junction and into the 3′ end of the *A. actinomycetemcomitans* insert DNA. Double-stranded plasmid DNA was sequenced using dye terminator chemistry and cycle sequencing using the BigDye terminator kit according to the manufacturer's instructions (ABI Perkin Elmer). The reactions were run on an ABI 377 sequencer.

Bioinformatics. The DNA sequences were analyzed using BLAST searches of the *A. actinomycetemcomitans* database at the University of Oklahoma (http://www.genome.ou.edu/act.html) and also using the PEDANT database, which contains complete and partial genome sequences of bacteria, including *A. actinomycetemcomitans* and *Haemophilus influenzae* (http://pedant.mips.biochem.mpg.de/). PEDANT was also the source of the numbering for the *A. actinomycetemcomitans* open reading frames (ORFs). The database of all derived protein sequences was also searched at the NCBI database. Segments containing the first 70 amino acids were searched for signal sequences using the SignalP program (http://www.expasy.ch/tools/). For those proteins that were negative on the SignalP programe, the transmembrane protein sequence analysis program DAS, TMpred, TMHMM, and HMMTOP on the Expasy Tools site (http://www.expasy.ch/tools/) were used.

Immunoscreening of *phoA* clones. A volume of 5 μl of each *phoA* clone was spotted onto nutrient agar containing ERY (150 μg/ml) and grown for 18 h at 37°C. Colony lifts were made onto 0.45-μm-pore-size nitrocellulose membranes (Nitrocellulose Extra; Sartorius), and these were blocked by immersion for 30 min in phosphate-buffered saline (PBS) containing 0.5% Tween 20 (PBS-T) and 5% skim milk powder. Membranes were washed three times in PBS-T for 10 min per wash. They were then incubated for 1 h at room temperature with immunoadsorbed rabbit antiserum (1:200 dilution) to the saline wash of *A. actinomycetemcomitans*. Membranes were then washed three times for 10 min each in PBS-T before being incubated for 1 h at room temperature in a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Sigma A2074). Following a further three washes in PBS-T, membranes were incubated with a commercial ECL Western blotting detection reagent (Amersham Pharmacia Biotech), and the enzymic reaction was determined by exposing the treated membranes to X-ray film.

Positive clones and analysis of insert sizes. A total of 80 clones, out of 8,000 screened, producing alkaline phosphatase fusion proteins were identified, with insert sizes ranging from 300 bp to 6 kb. Of these, 77 positive clones were sequenced.

Sequencing of clones. The protein sequences derived from the DNA sequences across the fusion junction were used in BLAST searches of the *A. actinomycetemcomitans* database and the full-length ORFs were obtained. The derived full-length protein sequence was then used in a BLAST search of all protein sequences at the NCBI database. Data analysis was confirmed by using the PEDANT database, which in most cases provided an annotation of the primary data from the University of Oklahoma *A. actinomycetemcomitans* genome database. The amount of protein fused to PhoA in each of the recombinants and the size of the full-length ORF, provided from genome databases, is shown in Tables 1 to 3. A number of the clones could not be sequenced, or the sequences showed that two different pieces of *A. actinomycetemcomitans* genomic

TABLE 2. *A. actinomycetemcomitans* clones showing homology to predicted *H. influenzae* proteins[a]

Clone[c]	No. of fused aa	ORF (full length)	Protein identity	% Similarity	% Identity	SP or TM
2	74	2007 (169)	HI1085	89	79	TM
10	82	437 (227)	HI1603	89	80	No
15	378	1091 (404)	HI1701	66	49	TM
21	31	2011 (191)	HI1150	74	62	SP
42	145	543 (269)	HI0693	89	78	SP
51	65	802 (204)	HI0370	74	51	TM
53[b]	43	(367)	HI1236	24	44	SP
57[b]	246	(371)	HI1126.1	87	80	TM
66	25	1301 (136)	HI1628	58	46	TM
67	101	748 (185)	HI0389	65	50	SP

[a] The table provides information on the number of amino acids in the protein fused to the PhoA, the ORF number (derived from the PEDANT database), the number of amino acids encoded by the full-length ORF, the identity of the protein, the identity to the same protein in the *H. influenzae* genome database, and whether the cloned protein has a signal peptide (SP) or transmembrane spanning (TM) segment.

[b] DNA sequence not yet in the PEDANT annotated *A. actinomycetemcomitans* database so no ascribed ORF number is available.

[c] Numbers in boldface type represent clones that were recognized by the rabbit antiserum to a saline wash of *A. actinomycetemcomitans*.

DNA had become fused to *phoA*, and were not interpretable. A number of clones revealed the presence of identical DNA sequences. For example four clones contained the signal sequence of peptide methionine sulphoxide reductase. Tables 1 to 3 and Fig. 1 show the 48 nonredundant clones identified in this study.

Identification of proteins fused to *phoA*. Proteins fused to *phoA* could be divided into four groups. The first were those proteins that had homology to known *H. influenzae* proteins (Table 1). The second were those proteins that had homology to hypothetical proteins in the *H. influenzae* database (Table 2). The third were those proteins which had no homology to *H. influenzae* proteins but could be recognized by homology to other bacterial proteins (Table 3). The final group consisted of those proteins that had no homology to any proteins in the current sequence databases (Fig 1).

Immunoscreening of *phoA* clones. A high-titer polyclonal antiserum to a saline wash of *A. actinomycetemcomitans* was used to immunoscreen the *phoA* clones. Negative controls of *E. coli* CC118 containing unligated vector, as expected, failed to bind the antiserum. This antiserum bound to 22 out of the 48 clones identified in this study (Fig 2). These antibody-binding clones are identified in the tables by the emboldened clone numbers. All four of the clones showing no homology to proteins in sequence databases were recognised by this antiserum (Fig 1).

The bacteria causing the periodontal diseases form biofilms between the teeth and the gums, in lesional sites called periodontal pockets, and there is limited contact between these organisms and the cells and tissues in which the disease is manifest (14). It is therefore assumed that the periodontal diseases are driven largely by secreted virulence factors emanating from the causative bacteria. We have attempted to isolate and identify the secreted proteins of *A. actinomycetemcomitans*, a major periodontopathogen (9, 17) by growing cells on agar and washing them with saline. Separating the extracted material by two-dimensional polyacrylamide gel electrophoresis revealed between 150 and 200 individual proteins (6). Isolating and identifying the virulence proteins in this population has proved to be difficult and, to date, we have only identified chaperonin 60 (6) as a putative virulence factor.

A generic strategy for the identification of at least a proportion of the genes encoding secreted bacterial proteins is based on the generation of translational fusions to a truncated gene for alkaline phosphatase (PhoA) lacking a functional signal sequence (5). Expression of alkaline phosphatase within the reducing environment of the cytoplasm is incompatible with correct folding and results in an inactive enzyme. It is only if the enzyme is translocated across the cytoplasmic membrane, into the oxidizing environment of the periplasm, that enzymic activity is generated. This can only occur if the DNA encoding the PhoA fuses with the coding regions of heterologous signal sequences or a transmembrane spanning sequence which allows the alkaline phosphatase to fold in the periplasm and be anchored to the cytoplasmic membrane. A genomic library of the bacterium of interest is prepared in the truncated *phoA*-containing vector and colonies are screened for the presence of alkaline phosphatase activity using a colorimetric substrate. Positive colonies can then be expanded, the plasmid can be isolated, and the DNA encoding the heterologous signal sequence can be sequenced.

In the present study, 8,000 colonies of a *phoA* fusion library of *A. actinomycetemcomitans* genomic DNA were screened, and 80 positive colonies were identified. Of these, 77 had

TABLE 3. *A. actinomycetemcomitans* clones showing homology to other bacterial proteins[a]

Clone[b]	No. of fused aa	ORF (full length)	Protein identity	% Similarity	% Identity	SP or TM
8	119	1058 (333)	*Methanosarcina barkeri* hypothetical protein	46	26	SP
12	66	1752 (478)	*E. coli* 46-kDa membrane protein	69	52	TM
34	98	793 (300)	*H. somnus* 31-kDa antigen gene	76	61	SP
40	132	1701 (690)	*E. coli* α-amylase precursor	72	60	SP
44	102	2059 (639)	*Y. pestis* conserved hypothetical protein TP0792	74	61	SP
55	80	1613 (4000)	*P. aeruginosa oprN* gene	45	27	SP
63	25	362 (143)	Cytolethal distending toxin protein C	100	100	SP
65	138	1787 (252)	*E. coli* hydrogenase isoenzyme formation protein HypB	81	70	No
74	45	770 (76)	*E. coli* YnbE protein precursor	73	52	SP
78	38	1745 (198)	*P. multocida* Skp 26 protein	73	64	SP

[a] The table provides information on the: number of amino acids in the protein fused to the PhoA, the ORF number (derived from the PEDANT database) the number of amino acids encoded by the full-length ORF, the identity of the protein, the identity and similarity to the same protein in the *H. influenzae* genome database, and whether the cloned protein has a signal peptide (SP) or transmembrane spanning (TM) segment.

[b] Numbers in boldface type represent clones that were recognized by the rabbit antiserum to a saline wash of *A. actinomycetemcomitons*.

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Clone 18 A.a ORF 313 [identified by rabbit antisera]
MKKFAKISTLLTACALAVQAYA⇓APLSIEKQGSFAVGGTVKTSEGTYTPIP
DAIKNRQSSAFFDVYGEAVKAGGMTLHGDHASVFYQIPTNAKQNSLVFLQ
GYGQSARGWMTTPDGHEGFNELFLQRNYPVYLVDQPRRGQAGRSTVDANV
PATPDDQFWYAQFRIGVYPKMNEGVAFPKDAESQAQFFRMMTPDTGAFDV
PVITDSMVKLFDKTSGGVFVTHSAGGVIGWTTAMASDKVKGVVAYEPGAF
FFPEGETPAKLESKFGDVAPLTVPKAQFEKLTKMPIVIYFGDFIPDHLDG
TQGGEQWFIRMKMAQQFVDTINKHGGKAELIHLPKIGIKGNTHFMFSDLN
NDKVAEEMARWLKAKGLDK

Clone 23 A.a ORF 551 [identified by rabbit antisera]
MKRANFPFRLTFLAAGLALLTACHSII⇓YQPAKTIEHIYLQSGYRLENVMQ
QALQKENLVIMTFSGGGSRAASLGYGVLEQFKNASVRPTEKGDTLLQNID
VVYGVSGGSVLAAYFAQEGQDVIPKFNESFLKKDFQKKVINEVFSMSNVP
RLTSPQFGRSDLLQEQLNLALYKGKKFADLAQHRKGPFAVINATDMAMGQ
KISFTQDFFDWLCLDLNDIEIARAVAASSAVPLIFSPVTLNNHGGSCHIH
NKKAMLTEQPGYWLLLNNFNAMEKRFARYQNNPEKTYLHLVDGGLTDNLG
LASLLDMSNLLSMHELYTELKKSNLRNIVVVNVNAQNENTSQIDKSADVP
GVKEVVNTVISVPIDKATESTLQYSQKFADQWNAYTKRKKDVKIKIYFVN
LSLRNLPEGQLKTDVLHIGTSFYLPESDVDKLREAAKILLEQSKEYQEAL
KALQ

Clone 26 A.a ORF 1440 [identified by rabbit antisera]
MYKQQFSFSDSGGQGKTTYINQNNGKKFDPKNAADVSELGKSIAFEVFEI
KENKDSHSVFESGAGICYGFKYTDGVAFTDSTTYYVDKSKQQYYASIIGA
TVSSDVEPKNVQYAPVFNIQDPELDKEVKSEEQRNGKTLINKNLQKSREI
LSNVVCK

Clone 33 A.a ORF 530 [identified by rabbit antisera]
MFKKLLMIIPLLVLTACSTGTASY⇓QPDQDEEQLNQLIVSQLKTKGSSRVV
LHHRQLLHFY

FIG. 1. Protein sequences of the four proteins with no homologies to other proteins in current sequence databases. A vertical arrow indicates the site of signal sequence cleavage using the SignalP V1.1 program. A horizontal arrow indicates that the C terminus of the protein is not known and the amino acid sequence shown is fused to the truncated PhoA at this point. Clone 26 ORF 1440 has no signal sequence cleavage site but has a weak membrane-spanning segment just prior to the point of fusion to PhoA. The clones which bound to the rabbit antisera raised to saline washes of *A. actinomycetemcomitans* have been identified.

inserts and were sequenced. The sequences of the *A. actinomycetemcomitans* DNA inserts (signal sequence plus variable amount of the ORFs of the individual genes) were compared to the genome sequence of *A. actinomycetemcomitans* to identify the full length ORFs. The sequencing of the genome of *A. actinomycetemcomitans* is almost completed (http://www.genome.ou.edu/act.html). These ORFs were then subject to further analysis to identify the nature of the genes. Of the 77 clones subject to sequence analysis, 72 gave unambiguous sequences, and when these sequences were analyzed 68 could be identified as full-length ORFs. A number of the clones contained the same or similar DNA sequences such that the total number of genes identified was 48. These proteins divide into those that can be ascribed a function based upon homology to proteins produced by other bacteria (34 proteins) and those that are either hypothetical proteins or proteins not found in any sequence databases (14 proteins). Of the 48 proteins identified, all bar three had either predicted signal peptides or a transmembrane spanning segment.

The assumption made in this and in other papers using the *phoA* cloning strategy is that the proteins identified are those exported in *E. coli*. In order to strengthen this assumption we have utilized a high-titer rabbit polyclonal antiserum to a saline wash of *A. actinomycetemcomitans* to immunoscreen the *phoA* clones. The saline wash contains protein secreted and loosely associated with the bacterial surface. The antiserum recognized a large proportion of the proteins in this saline wash fraction. The bacteria were not lysed during the immunoscreening and thus any colonies binding antibody must have had the recombinant protein or protein fragment associated with the cell surface. Of the 48 colonies screened, 22 bound the antiserum. This included all four of the proteins listed in Fig. 1, which, as yet, have no homology to proteins in sequence databases. This shows that these genes are encoding immunoreactive proteins. The antiserum also picked up a protein homologous to the aspartate aminotransferase of *H. influenzae*. This protein had no predicted signal sequence or transmembrane spanning region, which suggests that in spite of the absence of these characteristics this protein can gain access to the bacterial surface. The failure of the antiserum to recognise all clones is no doubt due to the fact that many of the inserts are small and therefore antigenic sites on these proteins are not expressed.

A recent report has also used the PhoA fusion methodology to identify secreted proteins of *A. actinomycetemcomitans* (8). These workers screened 2,500 colonies and found 28 alkaline phosphatase-positive colonies. Five of these clones proved to have homology to known proteins, and a number appear to be similar to the proteins identified in this study, such as the hypothetical *H. influenza* protein HI0370 (*A. actinomycetemcomitans* ORF 802). However, the identities of the other 23 proteins were not presented in this paper.

The proteins identified in this study as being exported/secreted include periplasmic binding proteins involved in nutrient transport, redox proteins such as MrsA (known to be involved in bacterial virulence [2, 18]) and DsbD and resistance

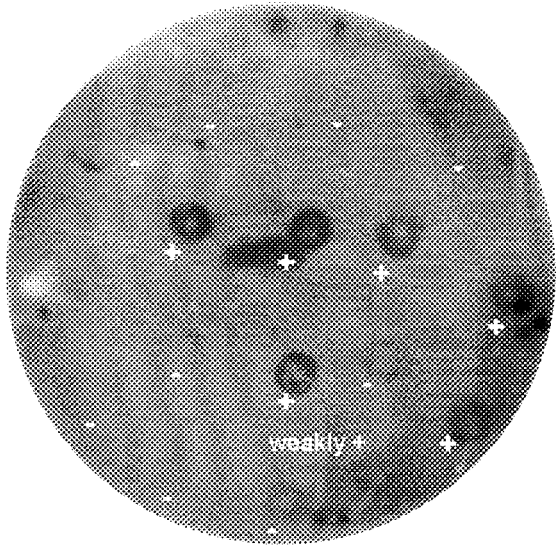

FIG. 2. Typical results of screening *phoA* clones with a polyclonal antiserum to a saline wash of *A. actinomycetemcomitans*. Some clones bind to the antibody (+) while others show no reaction (−). Clones which react with this antiserum have been denoted in the tables by emboldened clone numbers.

proteins (to organic solvents or antibiotics). One protein, CdtC is a toxin and putative virulence factor (11). Fourteen proteins have no ascribed function and would therefore be worth further study to see if they are involved in bacterial virulence.

The PhoA fusion cloning method described in this paper is a relatively rapid methodology for identifying secreted proteins in bacteria of interest. We have recently used it to identify secreted proteins in *Staphylococcus aureus* (15) and identified 18 novel genes, including a novel group of genes encoding exotoxins (16). Here we show the utility of the system for *A. actinomycetemcomitans* and describe four exported proteins (Fig. 1) not identified in current databases, of which all appear to be secreted. Further work is under way to determine if these proteins are involved in the virulence characteristics of *A. actinomycetemcomitans*.

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