

Additions and Corrections

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Page 16601: The labeled numbers in Fig. 3 were incorrect. The correct figure and legend are shown below.

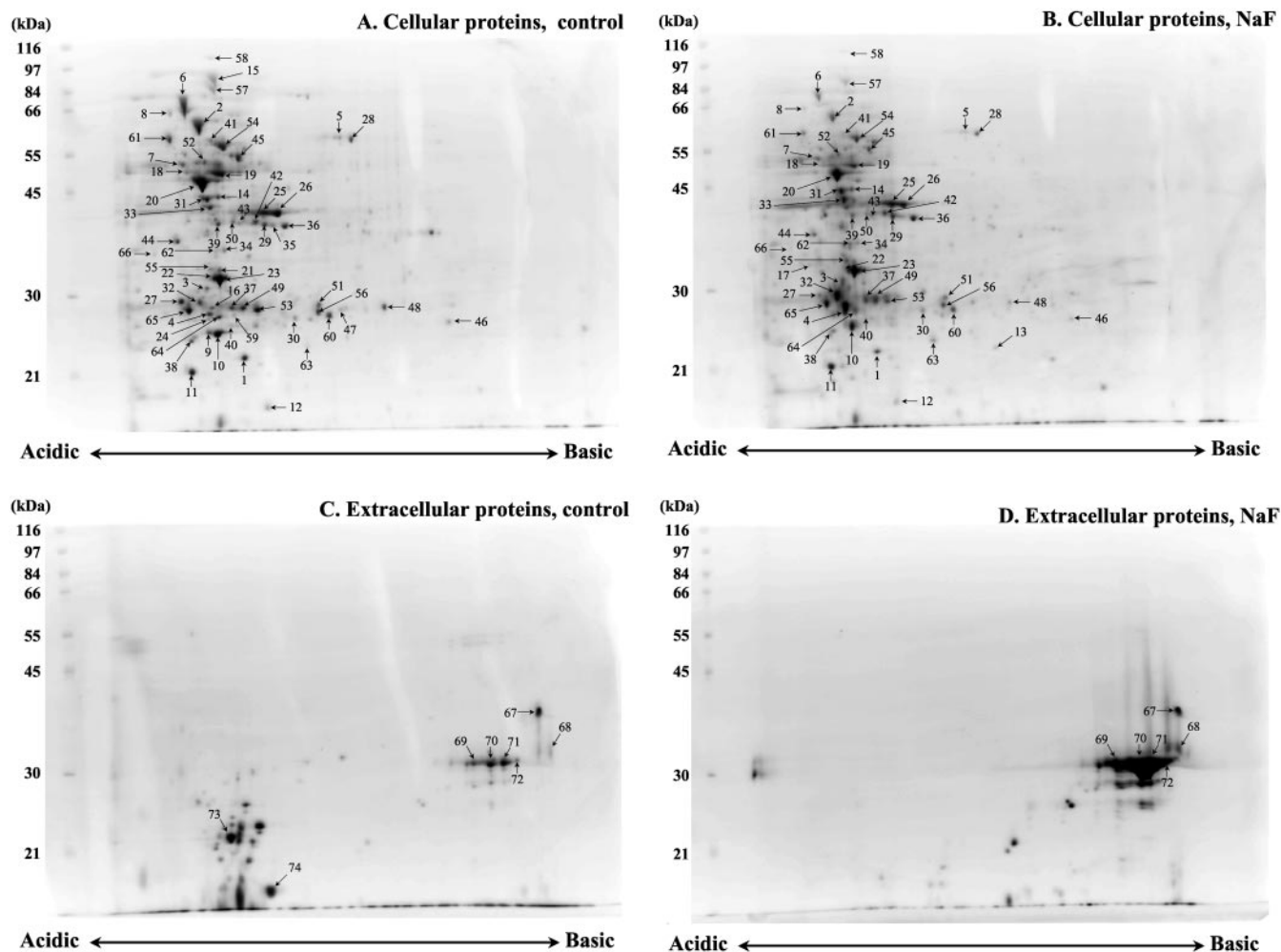


FIG. 3. Proteome maps for *S. pyogenes* and alterations by fluoride. The proteins were resolved by differential pI for the first dimension and by differential M_w for the second dimension of two-dimensional PAGE. Protein spots were visualized by SYPRO ruby staining, underwent in-gel tryptic digestion and MALDI-TOF mass spectrometry, followed by peptide mass fingerprinting using the MASCOT search engine. The significantly matched proteins (scores > 71, $p < 0.05$) are labeled as the same spot number as shown in Tables I and II.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.

Fluoride Exposure Attenuates Expression of *Streptococcus pyogenes* Virulence Factors*

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Fluoridation causes an obvious reduction of dental caries by interference with cariogenic streptococci. However, the effect of fluoride on group A streptococci that causes rheumatic fever and acute poststreptococcal glomerulonephritis is not known. We have used proteomic analysis to create a reference proteome map for *Streptococcus pyogenes* and to determine fluoride-induced protein changes in the streptococci. Cellular and extracellular proteins were resolved by two-dimensional polyacrylamide gel electrophoresis and identified by matrix-assisted laser desorption ionization mass spectrometry. 183 protein spots were visualized, and 74 spots representing 60 unique proteins were identified. A 16-h exposure to sodium fluoride caused decreased expression of proteins required to respond to cellular stress, including anti-oxidants, glycolytic enzymes, transcriptional and translational regulators, and protein folding. Fluoride caused decreased cellular expression of two well-characterized *S. pyogenes* virulence factors. Fluoride decreased expression of glyceraldehyde-3-phosphate dehydrogenase, which acts to bind fibronectin and promote bacterial adherence. We also performed proteomic analysis of protein released by *S. pyogenes* into the culture supernatant and observed decreased expression of M proteins following fluoride exposure. These data provide evidence that fluoride causes decreased expression by *S. pyogenes* proteins used to respond to stress, virulence factors, and implicated in non-suppurative complications of *S. pyogenes*, including glomerulonephritis and rheumatic fever.

glomerulonephritis (APSGN)¹ has decreased over the last five decades in the United States and western countries (1–3). However, these disorders continue unabated and are important public health problems in developing countries (1, 3, 4). The effects of fluoride on cariogenic *Streptococcus mutans* and other bacteria have been extensively studied. Fluoride inhibits enolase, a glycolytic enzyme (5, 6), inhibits F-ATPase activity resulting in less acidurance (7, 8), reduces glucan-binding lectin activity (9), and decreases glucose incorporation (10). However, the effects of fluoride on group A Streptococci have not been examined. To examine simultaneous changes in multiple virulence factors, we performed a proteomic analysis of *S. pyogenes* exposed to fluoride. Western blotting and other immunological methods have been successfully used to study protein expression of various microorganisms, cells, and tissues. However, these techniques are constrained by the limited number of proteins that can be studied in each experiment and the availability of specific antibodies. Proteomic techniques date to 1975, when two-dimensional PAGE was simultaneously described by O'Farrell and Klose (11, 12) and applied to the study of a large number of proteins simultaneously. In two-dimensional PAGE proteins are separated by differential isoelectric point (pI) for the first dimension and by differential weight average molecular weight (M_w) for the second dimension. Using this technique, 1100 protein components have been resolved from *Escherichia coli* (11). Recently, up to 10,000 protein forms have been visualized by high resolution two-dimensional PAGE (12). The high throughput analysis by mass spectrometry of proteins separated with two-dimensional PAGE has permitted analysis of proteins on a "genomic" scale (13), a process that has acquired the name "proteomics" (14). We have used this approach to construct an initial proteome map of *S. pyogenes* and to determine whether fluoride alters protein expression of *S. pyogenes*. In this initial analysis, 74 spots representing 60 unique proteins were identified. Fluoride caused *S. pyogenes* to decrease expression of several virulence factor proteins, including M protein, GAPDH, and deoxythymidine diphosphate (dTDP)-4-keto-6-deoxyglucose-3,5-epimerase, but did not alter cell viability. These proteomic data

The incidence of some sequelae of group A streptococcal infection such as rheumatic fever and acute post-streptococcal

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||| Posthumous.

¹ The abbreviations used are: APSGN, acute poststreptococcal glomerulonephritis; pI, isoelectric point; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; CDM, chemically defined medium; DTT, dithiothreitol; α -CN, α -cyano-4-hydroxycinnamic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M_w , weight average molecular weight; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HSP, heat-shock protein.

suggest the hypothesis that fluoride might inhibit *S. pyogenes* virulence factors and post-infectious inflammatory disorders.

MATERIALS AND METHODS

Bacteria and Growth Conditions—*S. pyogenes* M5 was employed throughout this study. The streptococci were grown either in chemically defined medium (CDM) (15) as a control or in CDM with 5 mM sodium fluoride (NaF, "Suprapure"; E. Merck, Darmstadt, Germany) at 37 °C for 16 h. NaF was separately filter-sterilized before adding into the sterile medium.

Protein Extraction—Bacteria were harvested after overnight culture by centrifugation at 6000 × *g* at 4 °C for 10 min. Both cellular proteins and proteins in the extracellular supernatant were extracted. For the cellular components, the bacteria were washed twice with ice-cold 18-megohm water and then sonicated at level 4 with 60% duty cycle (High Intensity Ultrasonic Cell Disrupter, Sonics & Materials Inc, Danbury, CT) on ice until more than 80% of cells were broken. The protein mixture was centrifuged at 6000 × *g* at 4 °C for 10 min, and the supernatant was saved. The sample was lyophilized and resuspended in a sample buffer containing 40 mM Tris, 7.92 M urea, 0.06% SDS, 1.76% ampholytes, 120 mM dithiothreitol (DTT), 3.2% Triton X-100, 0.1 mg/ml leupeptin, 0.1 mg/ml phenylmethylsulfonyl fluoride, and 1 mM sodium azide.

For the extracellular proteins, the culture supernatant was precipitated overnight by ammonium sulfate and centrifuged at 25,000 × *g* at 4 °C for 30 min. The pellet was saved, resuspended in 20 mM phosphate-buffered saline (pH 7.2) and dialyzed two times against 18-megohm water with the M_w cut off at 6–8 kDa overnight. The samples were lyophilized and resuspended in the same sample buffer as above.

The samples were duplicated and protein concentration was measured by spectrophotometry using Bio-Rad protein microassay based on Bradford's method (16).

Two-dimensional PAGE—The control and NaF-treated samples were run in parallel with a two-dimensional PAGE running system (Genomic Solutions Inc., Ann Arbor, MI).

First Dimension—Immobilized pH gradient strips, non-linear pH 3–10, 18 cm long (Amersham Biosciences, Inc., Fairfield, NJ) were rehydrated overnight with 100 μg of proteins in rehydration buffer containing 8 M urea, 2% CHAPS, 0.01 M DTT, 2% ampholytes, and bromphenol blue and focused with maximal 5000 V and 80 μA for 24 h at 17 °C to reach 100,000 V.h. After completion of focusing the samples were equilibrated with buffer containing 6 M urea, 130 mM DTT, 30% glycerol, 112 mM Tris base, 4% SDS, 0.002% bromphenol blue and acetic acid and then with buffer containing 6 M urea, 135 mM iodoacetamide, 30% glycerol, 112 mM Tris base, 4% SDS, 0.002% bromphenol blue, and acetic acid.

Second Dimension—The strips were loaded onto pre-cast 10% homogeneous, 20 × 20-cm slab gels (Genomic Solutions Inc.). Upper running buffer contained with 0.2 M Tris base, 0.2 M Tricine, and 0.4% SDS and lower running buffer was 0.625 M Tris acetate. The system was run with maximal 500 V and 20,000 milliwatts per gel.

SYPRO Ruby Staining—The gel slabs were fixed in 10% methanol and 7% acetic acid for 30 min. The fixed solution was removed and 500 ml of SYPRO ruby gel stain (Bio-Rad Laboratory, Hercules, CA) was added to each gel and incubated on gently continuous rocker at room temperature for 18 h.

Visualization—A high resolution 12-bit camera with UV light box system (Genomic Solutions Inc.) was used to visualize the protein spots. Five different exposure time points (1, 2, 3, 4, and 5 s) were set to scan the gels. The images were inverted before analysis with two-dimensional analysis software.

Matching and Analysis of the Protein Spots—Investigator HT analyzer (Genomic Solutions Inc.) software was used for matching and analysis of the protein spot expression on gels. A reference gel was created by combining all of the spots from different gels into one image. The average mode of background subtraction was used for normalization of intensity volume of each spot and for compatibility of the intensity between each gel. The reference gel was then used for determination of existence and difference of protein expression between each group. The intensity less than a 0.5-fold or greater than 2-fold of the control was considered significantly changed.

In-gel Tryptic Digestion—Samples were prepared using a modification of the technique described by Jensen (17). The protein spots were excised with a clean scalpel into 1-mm cubes. The gel pieces were transferred to clean 1.5-ml microcentrifuge tubes and wash with 0.1 M ammonium bicarbonate (NH₄HCO₃) at room temperature for 15 min. Acetonitrile was added to the gel pieces and incubated at room temper-

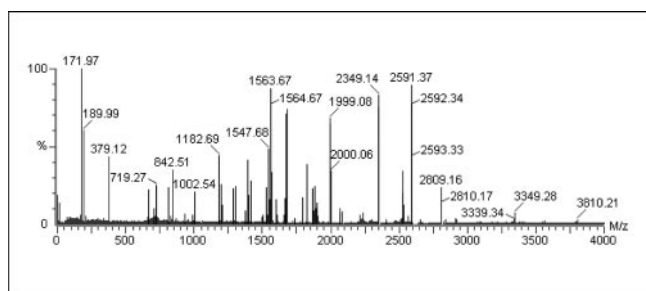


FIG. 1. MALDI-TOF mass spectrometry. Illustrates an example of peptide masses obtained by MALDI-TOF mass spectrometry that is typical for the mass spectra of 60 kDa chaperone (GroEL) from spot number 2 in Fig. 3.

g|13959318: 60 kDa chaperone; Locus CH60_STRPY; Source *S. pyogenes*; Score 399; Coverage 68%

Observed	Theoretical	Delta	Start - End	Miss	Peptide
665.28	665.31	-0.04	7 - 12	0	FSADAR
718.26	718.34	-0.08	276 - 282	0	APGFGDR
812.44	812.44	0.00	443 - 449	0	ALEEPVR
933.53	933.53	-0.00	267 - 275	0	GTFNVAVK
985.50	985.54	-0.05	379 - 388	0	VGAPTFALK
1001.53	1001.51	0.02	394 - 402	0	IEDALNATR
1181.68	1181.65	0.03	105 - 116	0	NVTAGANPIGIR
1200.68	1200.65	0.03	141 - 152	0	EALQVAAVSSR
1282.63	1282.59	0.04	156 - 166	0	VGEYISEAMER
1298.59	1298.58	0.01	156 - 166	0	VGEYISEAM*ER
1373.71	1373.72	-0.02	379 - 391	1	VGAPTFALKEMK
1387.72	1387.69	0.02	167 - 179	0	VGNDGVITIEESR
1398.66	1398.63	0.03	349 - 360	0	SQLETTTDFDR
1415.70	1415.69	0.01	424 - 437	0	VAALELEGGDATGR
1529.86	1529.84	0.02	117 - 131	1	RGIETATATAVEALK
1546.67	1546.70	-0.02	196 - 208	0	GYLSQYMVTDNEK
1562.66	1562.69	-0.03	196 - 208	0	GYLSQYM*VTDNEK
1602.86	1602.86	-0.00	42 - 57	0	AFGPSPLITNDGVTIK
1660.74	1660.74	-0.00	58 - 71	0	EIELEDHFENMGAK
1675.86	1675.88	-0.02	450 - 465	0	QIALNAGYEGSVVYDK
1733.85	1733.89	-0.04	209 - 223	0	M*VADLENPFILITDK
1790.89	1790.86	0.03	326 - 343	0	DSTVIVEGSGSSEAIANR
1822.00	1822.05	-0.05	225 - 240	0	VSNIQDILPLEEVLK
1868.84	1868.83	0.02	180 - 195	0	GMETELEVVEGMQFDR
1884.81	1884.82	-0.02	180 - 195	0	GM*ETELEVVEGMQFDR
1900.78	1900.82	-0.04	180 - 195	0	GM*ETELEVVEGM*QFDR
1997.06	1997.10	-0.05	403 - 423	0	AAVEEGIVAGGGTALITVIEK
2064.93	2064.96	-0.03	468 - 487	0	NSPAGTGFNAATGEWVDMIK
2347.14	2347.19	-0.05	321 - 343	1	ITVDKDSSTVIVEGSGSSEAIANR
2525.21	2525.30	-0.09	80 - 104	0	TNDIAGDGTATVLTQAIHVHGLK
2530.21	2530.35	-0.14	285 - 308	0	AM*LEDIALITGGVITIEDLGLK
2589.34	2589.44	-0.10	241 - 264	0	TNRPLLIADDVDEGALPTLVLNK

No match to: 709.34, 1506.25, 2407.99

FIG. 2. Peptide mass fingerprinting. Peptide mass fingerprinting of the observed masses in Fig. 1 was performed using the MASCOT search engine. Scores more than 71 were considered statistically significant for matching ($p < 0.05$). Observed masses (32 of total 35 masses) were matched to the theoretical masses of 60 kDa heat shock protein (GroEL) with less than 150-ppm window of error and mostly 0 missed cleavage. The matched masses were then converted to amino acid sequences along variable residue sites and covered 68% of the GroEL sequences. * An oxidation site on methionine that caused mass shift.

ature for 15 min. The solvent was removed, and the gel pieces were dried in laminar flow hood. The gel pieces were rehydrated with 20 μl of 20 mM DTT in 0.1 M NH₄HCO₃ and incubated at 56 °C for 45 min to reduce the protein. The tubes were chilled at room temperature, and the DTT solution was removed and replaced with 20 μl of 55 mM iodoacetamide in 0.1 M NH₄HCO₃ and incubated at room temperature in the dark for 30 min. The iodoacetamide was removed and replaced with 0.2 ml of 50 mM NH₄HCO₃ and incubated at room temperature for 15 min. Acetonitrile (0.2 ml) was added, and the samples were incubated at room temperature for 15 min. The solvent was removed, and the gel pieces were dried in laminar flow hood. The gel pieces were rehydrated with 20 ng/μl modified trypsin (Promega, Madison, WI) in 50 mM NH₄HCO₃ with the minimal volume to cover the gel pieces. The gel

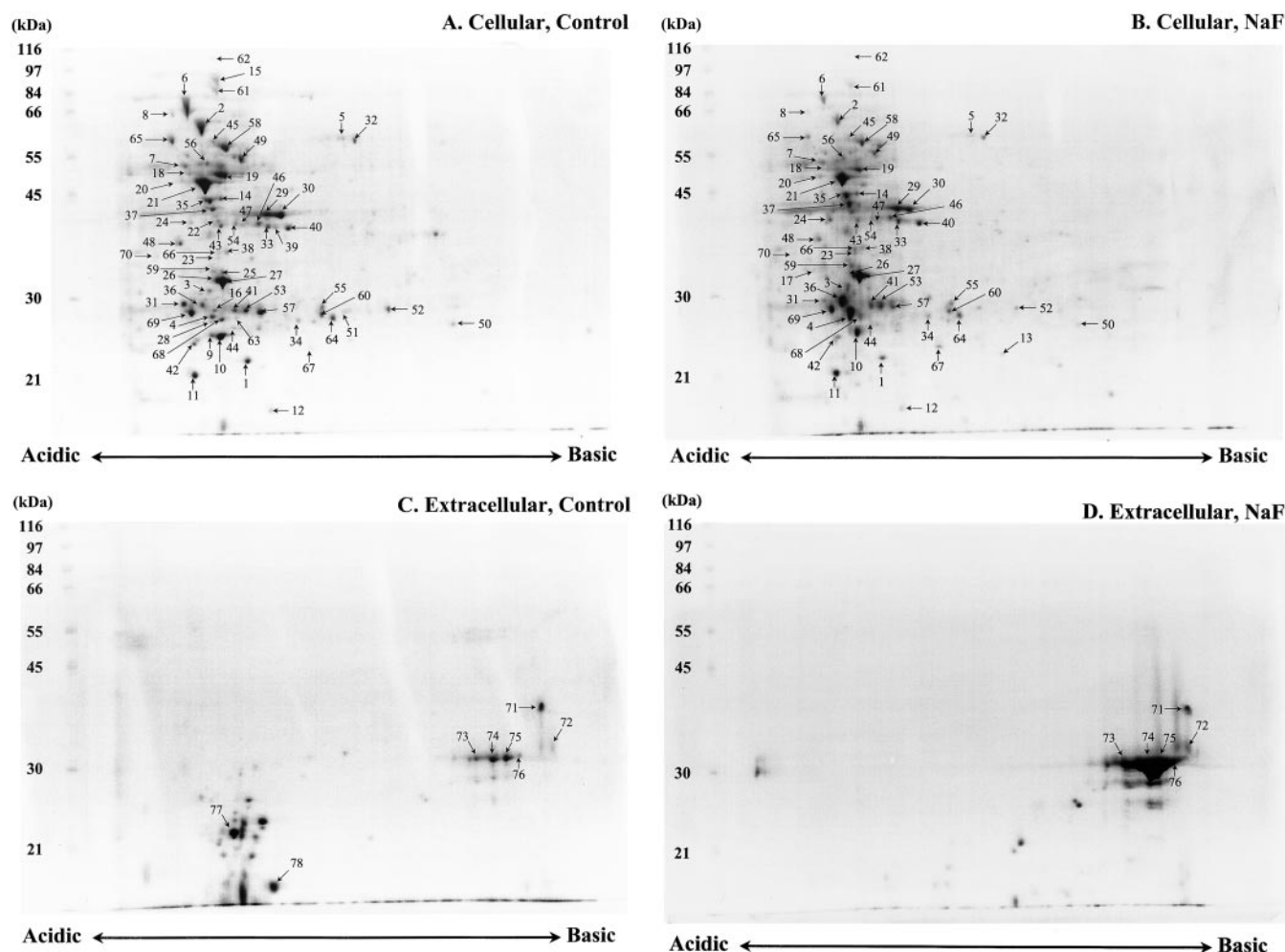


FIG. 3. Proteome maps for *S. pyogenes* and alterations by fluoride. The proteins were resolved by differential pI for the first dimension and by differential M_w for the second dimension of two-dimensional PAGE. Protein spots were visualized by SYPRO ruby staining, underwent in-gel tryptic digestion, MALDI-TOF mass spectrometry and followed by peptide mass fingerprinting using the MASCOT search engine. The significantly matched proteins (scores > 71, $p < 0.05$) are labeled as the same spot number as shown in Table I and II.

pieces were chopped into four to five smaller pieces and incubated at 37 °C overnight in shaking incubator to enhance microcirculation of the digestive solution and to prevent drops formation under the cover of microcentrifuge tubes.

Sample Preparation for MALDI-TOF Mass Spectrometry—Nitrocellulose solution was made by dissolving a nitrocellulose membrane in 1:1 acetone/isopropanol solvent. α -Cyano-4-hydroxycinnamic acid (α -CN) was washed with 50 μ l of acetone, and acetone phase was discarded. The α -CN was dissolved in acetone to a concentration of 10 mg/ml, and the nitrocellulose and α -CN solutions were mixed to 1:4 ratio, and 1 μ l of this mixture was deposited onto the 96-well MALDI target plate. The samples were prepared for addition to the plate by mixing 2 μ l of sample with 2 μ l of 10 mg/ml α -CN solution in 0.1% trifluoroacetic acid in 1:1 H₂O/acetonitrile. The sample mixtures (1 μ l) were loaded onto each thin film. After the sample mixtures were dried, 1.5 μ l of 2% formic acid was added to each spot. The formic solution was removed by gentle blotting. This washing step was performed twice. The samples were then dried at room temperature. Fragment size was determined by MALDI-TOF mass spectrometry.

MALDI-TOF Mass Spectrometry—Mass spectral data were obtained using a Micromass Tof-Spec 2E instrument equipped with a 337-nm N₂ laser at 20–35% power in the positive ion reflectron mode. Spectral data were obtained by averaging 10 spectra each of which was the composite of 10 laser firings. The mass axis was calibrated using known peaks from tryptic autolysis.

Analysis of Peptide Sequences—Peptide mass fingerprinting was used for protein identification from tryptic fragment sizes by using the MASCOT search engine (www.matrixscience.com) based on the entire NCBI protein data base using the assumption that peptides are monoisotopic, oxidized at methionine residues and carbamidomethylated at cysteine residues. Up to one missed trypsin cleavage was al-

lowed, although most matches did not contain any missed cleavages. Mass tolerance of 150 ppm was the window of error to be allowed for matching the peptide mass values. Probability-based MOWSE scores were estimated by comparison of search results against estimated random match population and were reported as $-10 \cdot \log_{10}(P)$, where P is the absolute probability. Scores greater than 71 were considered significant ($p < 0.05$).

RESULTS

***S. pyogenes* Cellular Protein Expression**—The pattern of protein separation by two-dimensional PAGE was consistent and essentially identical in different culture samples of *S. pyogenes*. A total of 183 cellular protein spots were visualized. The protein spots were excised and underwent in-gel tryptic digestion. Peptide masses were obtained by MALDI-TOF mass spectrometry. Shown in Fig. 1 is a typical mass spectra of a protein, the 60-kDa chaperone that was identified using the MASCOT search engine to query the NCBI protein data base (Fig. 2). All protein identifications were in the expected size range based on position in the gel. Fifty-five unique proteins were identified from 66 spots present on gels. (Fig. 3A, Table I).

Effect of Fluoride on *S. pyogenes* Cellular Protein Expression—Expression of 38 protein forms was decreased after exposure to fluoride and six protein forms had increased expression after fluoride exposure (Fig. 3B, Table I). General stress protein 24, elongation factors G and P, fructose-bisphosphate aldolase, putative 6-phosphofruktokinase, putative orotidine-5'-decarboxylase PyrF, and putative xanthine phosphoribosyl-

TABLE I
Cellular protein expression in *S. pyogenes*

Only significantly matched proteins by peptide mass fingerprinting are included. The identifier of each protein is the GenInfo number in the NCBI protein database. M_w and pI indicate the theoretical position of each protein spot on two-dimensional gel based on the protein database. Several proteins, for example fructose-bisphosphate aldolase, chaperone protein dnaK, and putative orotidine-5'-decarboxylase PyrF are expressed as multiple isoforms with similar pI and M_w on the gel.

Spot no.	Protein	ID no.	Coverage	pI	M_w	Intensity (NaF/control)
			%		kDa	
1	50 S ribosomal protein L10	gi 13959465	43	5.2	17.55	0.45
2	60-kDa chaperone (protein Cpn60) (GroEL protein)	gi 13959318	68	4.7	57.08	0.13
3	Adenylate kinase (ATP-AMP transphosphorylase)	gi 14194884	28	4.8	23.74	1.84
4	Calcium binding protein A	gi 11691918	28	4.4	19.37	7.63
5	Chain A, inosine monophosphate dehydrogenase	gi 7546367	16	5.6	50.89	0.34
6	Chaperone protein dnaK (heat-shock protein 70) (HSP70)	gi 3122027	49	4.6	64.90	0.18
7	Chaperone protein dnaK (heat-shock protein 70) (HSP70)	gi 3122027	32	4.6	64.90	0.50
8	Conserved hypothetical protein	gi 13622918	31	4.4	59.18	0.14
9	Conserved hypothetical protein ^a	gi 13622389	34	4.9	19.93	0.00
10	Conserved hypothetical protein ^a	gi 13622389	38	4.9	19.93	0.68
11	Conserved hypothetical protein [c] ^a	gi 13622387	75	4.6	17.44	0.85
12	Conserved hypothetical protein [d] ^b	gi 13622828	36	6.3	18.89	0.53
13	Deoxyuridine 5'-triphosphate nucleotidohydrolase, putative	gi 14971486	19	5.3	15.83	N.A. ^c
14	DNA-directed RNA polymerase alpha subunit	gi 13621394	30	4.9	34.57	0.54
15	Elongation factor G (EF-G)	gi 13959328	14	4.8	76.57	0.00
16	Elongation factor P (EF-P)	gi 13959327	21	4.9	20.45	0.00
17	Elongation factor Tu	gi 14578902	17	4.6	29.51	N.A.
18	Elongation factor TU (EF-TU)	gi 14194714	34	4.9	43.84	0.25
19	Elongation factor TU (EF-TU)	gi 14194714	49	4.9	43.84	0.23
20	Enolase (2-phosphoglycerate dehydratase)	gi 13959354	58	4.7	47.40	0.52
21	Fructose-bisphosphate aldolase	gi 14194455	27	4.9	31.30	0.00
22	Fructose-bisphosphate aldolase	gi 14194455	30	4.9	31.30	0.81
23	Fructose-bisphosphate aldolase	gi 14194455	58	4.9	31.30	0.90
24	Fructose-bisphosphate aldolase	gi 14194455	25	4.9	31.30	0.00
25	GAPDH (plasminogen-binding protein) (plasmin receptor)	gi 14195645	52	5.3	36.04	0.75
26	GAPDH (plasminogen-binding protein) (plasmin receptor)	gi 14195645	31	5.3	36.04	0.46
27	Heat-shock protein GrpE	gi 14971990	25	4.6	19.95	0.17
28	Inosine-5'-monophosphate dehydrogenase (IMPDH)	gi 1708474	29	5.6	52.85	0.54
29	Mannose-specific phosphotransferase system component IIAB	gi 13622792	25	5.2	35.55	1.19
30	M-like protein precursor	gi 5002352	10	5.6	39.73	1.14
31	Phosphoglycerate kinase	gi 14195003	63	4.8	42.11	0.64
32	Protein-tyrosine-phosphatase	gi 10176393	38	4.6	18.01	4.64
33	Putative elongation factor TS	gi 13623082	39	4.9	37.21	1.12
34	Putative 2-dehydropanoate 2-reductase	gi 13622023	8	4.8	34.15	0.33
35	Putative 6-phosphofructokinase	gi 13622406	28	5.3	35.73	0.00
36	Putative 6-phosphofructokinase	gi 13622406	71	5.3	35.73	0.98
37	Putative ABC transporter (ATP-binding protein)	gi 13622437	27	5.0	27.20	0.94
38	Putative alkyl hydroperoxidase	gi 13623068	40	4.7	20.64	0.12
39	Putative branched-chain-amino-acid aminotransferase	gi 13622075	49	4.9	37.10	0.70
40	Putative dTDP-4-keto-6-deoxyglucose-3,5-epimerase	gi 13622099	30	5.1	22.48	0.45
41	Putative GMP synthase	gi 13622332	18	4.9	57.71	0.25
42	Putative L-lactate dehydrogenase	gi 13622288	12	5.1	35.37	0.56
43	Putative L-lactate dehydrogenase	gi 13622288	9	5.1	35.37	0.60
44	Putative manganese-dependent inorganic pyrophosphatase (intrageneric coaggregation relevant adhesin)	gi 13621631	59	4.5	33.60	0.53
45	Putative NADP-dependent GAPDH	gi 13622481	47	5.1	50.41	0.24
46	Putative orotate phosphoribosyltransferase	gi 13622065	55	6.4	22.73	0.47
47	Putative orotidine-5'-decarboxylase PyrF	gi 13622064	35	5.9	25.07	0.00
48	Putative orotidine-5'-decarboxylase PyrF	gi 13622064	39	5.9	25.07	0.16
49	Putative phosphoglycerate mutase	gi 13622525	37	5.1	26.12	1.04
50	Putative phosphotransacetylase	gi 13622266	29	5.0	35.86	0.26
51	Putative polypeptide deformylase	gi 13622977	60	5.5	22.96	0.32
52	Putative proton-translating ATPase, beta subunit	gi 13621938	20	4.7	51.04	0.63
53	Putative pyrimidine regulatory protein	gi 13622002	62	5.2	19.50	0.14
54	Putative pyruvate kinase	gi 13622405	57	5.0	54.57	0.34
55	Putative pyruvate kinase	gi 13622405	15	5.0	54.57	1.34
56	Putative TctR-family transcriptional regulator	gi 13092852	11	5.6	25.09	0.46
57	Putative transketolase	gi 13622740	32	5.0	77.49	0.26
58	Putative transport system permease protein	gi 13363868	5	5.3	111.71	0.16
59	Putative xanthine phosphoribosyltransferase	gi 13622272	11	5.2	20.98	0.00
60	Ribosome recycling factor (ribosome releasing factor)	gi 14195167	43	5.7	20.55	0.71
61	RopA	gi 3549287	42	4.4	47.08	0.14
62	SigmaB regulating protein RsbU	gi 13701862	15	5.4	38.58	2.34
63	Subtilisin Dy	gi 135020	15	7.1	27.42	5.62
64	Superoxide dismutase [Mn]	gi 13959576	29	4.9	22.65	0.37
65	Triosephosphate isomerase	gi 13959585	39	4.6	26.89	0.91
66	yomF	gi 2634558	16	4.7	31.90	0.13

^a 61% of sequence is identical with the sequence of general stress protein 24 (gi|14973303).

^b 62% of sequence is identical with the sequence of universal stress protein family (gi|14973504).

^c N.A., not applicable (divided by zero).

TABLE II
Protein expression in *S. pyogenes* culture supernatants

Only proteins significantly matched by peptide mass fingerprinting are included. The identifier of each protein is the GenInfo number in the NCBI protein database. \bar{M}_w and pI indicate the theoretical position of each protein spot on two-dimensional gel based on the protein database.

Spot no.	Protein	ID no.	Coverage	pI	\bar{M}_w	Intensity (NaF/control)
			%		kDa	
67	Pyrogenic exotoxin B	gi 431620	38	8.8	43.14	0.78
68	RNA polymerase beta subunit	gi 6449117	16	7.9	19.30	0.90
69	Cysteine protease SpeB	gi 14699964	23	7.2	37.38	4.45
70	Cysteine protease SpeB	gi 14699964	40	7.2	37.38	5.23
71	Cysteine protease SpeB	gi 14699964	26	7.2	37.38	3.77
72	Cysteine protease SpeB	gi 14699964	17	7.2	37.38	7.92
73	Tetavalent M protein	gi 408225	9	5.3	28.23	0.00
74	M5 protein—streptococcal pyogenes (fragment)	gi 437191	10	6.0	22.70	0.00

transferase were absent after fluoride exposure. Deoxyuridine 5'-triphosphate nucleotidohydrolase was expressed only after fluoride exposure. Table III summarizes the differential expression of proteins that were classified based on their functional categories as modified from the functional categories of M1 *S. pyogenes* genome (18). Some of the proteins have multiple functions in bacteria, for example GAPDH is necessary for glycolysis, cell wall adhesion, and signal transduction. As shown in Table III, marked decreases in expression were seen in protein chaperones, general stress protein, regulators of DNA and RNA synthesis, glycolytic and other metabolic enzymes, and proteins essential to translation.

Protein Expression in *S. pyogenes* Culture Supernatants—A total of 62 protein spots were visualized and 8 spots representing 5 unique proteins were identified in culture supernatants (Fig. 3C, Table II). The identified proteins in culture supernatants were not observed in cellular components and have been previously described as extracellular proteins in streptococcal culture supernatants (19–21). Proteins present in culture supernatants were presumably from surface-expressed proteins and were released or shed during normal and stress conditions. Cysteine protease SpeB, also known as pyrogenic exotoxin B, was expressed as multiple forms on the two-dimensional PAGE that reflect the previously described cleavage of this protein (22). Two proteins identified in the data base as tetavalent M protein and M5 protein were present in culture supernatants.

Effect of Fluoride on Protein Expression in *S. pyogenes* Culture Supernatants—The active low molecular weight cysteine protease SpeB forms in supernatants had markedly increased expression, but the highest molecular mass form (~38 kDa) did not change expression. Notably, the expression of both M virulence forms identified as tetavalent M and M5 proteins were absent after fluoride exposure (Fig. 3D, Table II).

DISCUSSION

We have constructed an initial proteome map for both cellular and supernatant protein expression of *S. pyogenes*. This map permits consistent identification of proteins as the coordinates of the protein spots are highly reproducible on high resolution two-dimensional PAGE gels. We identified 60 unique cellular and culture supernatant proteins by peptide mass fingerprinting that were expressed in 74 forms on two-dimensional PAGE. Fluoride exposure markedly altered both intracellular protein expression and the content of proteins in the culture supernatant, without affecting cell viability. The altered proteins were summarized in Table III, and their functions were classified as modified from the function categories of M1 *S. pyogenes* genome (18).

Fluoride caused decreased expression of several proteins that have been implicated in *S. pyogenes* virulence. Fluoride caused decreased expression in culture supernatants of the well-characterized M Protein virulence factors. Decreased re-

lease of M protein into the culture supernatant presumably reflects decreased surface expression, because M protein is primarily confined to the bacterial membrane and intracellular expression of M protein was unchanged. M protein has been shown to promote *S. pyogenes* virulence by inhibiting phagocytosis and increasing adherence to host tissues. GAPDH expression was also decreased by fluoride. GAPDH expressed on the *S. pyogenes* cell surface acts as a virulence factor by binding fibronectin and stimulating signal transduction in host cells (23–25). Purified GAPDH stimulated serine and tyrosine kinase activity in host cells that was required for uptake of bacteria. Considered together, fluoride-induced decreases in M protein and GAPDH could result in decreased adherence to and penetration of the host epithelial barrier.

Paradoxically, we observed increased release into culture supernatant of the major streptococcal cysteine protease SpeB. The potential role of SpeB as a virulence factor has been studied extensively. SpeB activates interleukin-1 β , kininogen, and matrix metalloproteinases, presumably promoting inflammation and tissue destruction (26–28). Based on these findings, an increase in the release of SpeB would presumably promote virulence. However, studies that addressed the clinical relevance of SpeB as a virulence factor in animal models of infection have yielded conflicting results. Talkington *et al.* (29) observed no relation between SpeB expression and invasive *S. pyogenes* infection. Chaussee *et al.* (30) determined SpeB production in 117 *S. pyogenes* clinical isolates and observed no correlation with the severity of disease. Kansal *et al.* (31) observed an inverse relationship between disease severity and SpeB expression in *S. pyogenes* isolates from patients with invasive Group A infections. Finally, SpeB activity is governed, in part, by the protein RopA. RopA contributes to the post-translational processing of SpeB that establishes an active conformation after secretion (32). Fluoride caused a marked decrease in the expression of RopA. Considered together, these data suggest that, although SpeB may function as a virulence factor, changes in the absolute levels of SpeB expression may be less relevant to virulence.

Fluoride exposure did not directly affect cell viability but did alter the expression of proteins essential to survival and the response to stress. *S. pyogenes* exposed to fluoride expressed lower levels of GroEL and DnaK chaperone proteins, as well as general stress protein 24. Fluoride-treated *S. pyogenes* also had markedly decreased expression of proteins required for scavenging oxygen radicals. However, fluoride exposure caused increased expression of one protein that regulates the response to stress, RsbU. We observed a 2-fold increase in a protein with significant homology to RsbU. RsbU regulates the SigmaB protein that has been well characterized in the response of *Bacillus subtilis* to stress induced by heat, ethanol, salt, and energy starvation. Activation of the SigmaB regulon results in

TABLE III
Summary of the altered proteins after fluoride exposure

All of the proteins in both cell extracts and culture supernatants that were significantly regulated by fluoride were summarized and classified into several categories based on their functions in the bacteria. Most of the proteins were down-regulated or absent whereas few of them were up-regulated after fluoride exposure.

Functional categories	Protein	Spot no.	Regulation by NaF
Heat-shock protein/chaperone	60-kDa chaperone (GroEL)	2	Down
	Heat-shock protein 70 (dnaK)	6, 7	Down
	Heat-shock protein GrpE	27	Down
Other stress proteins	Superoxide dismutase (SOD) ^a	64	Down
	Alkyl hydroperoxidase ^a	38	Down
	General stress protein 24	9	Disappeared
DNA and RNA synthesis	Putative GMP synthase	41	Down
	Orotate phosphoribosyl transferase	46	Down
	Orotidine-5'-decarboxylase PyrF	47, 48	Down
	Chain A, Inosine monophosphate dehydrogenase	5	Down
	Pyrimidine regulatory protein	54	Down
	Xanthine phosphoribosyltransferase	59	Disappeared
	Deoxyuridine 5'-triphosphate nucleotidohydrolase	13	Newly expressed
Energy production and glycolytic pathway	Fructose-bisphosphate aldolase	21, 24	Disappeared
	GAPDH ^a	26	Down
	NADP-dependent glyceraldehyde 3-phosphate dehydrogenase ^a	45	Down
	6-Phosphofructokinase	35	Disappeared
	2-Dehydropantoate 2-reductase	34	Down
	Pyruvate kinase	54	Down
	Transketolase	57	Down
Transcription regulator	Putative TetR-family transcriptional regulator	56	Down
	RopA	61	Down
	SigmaB regulating protein RsbU	62	Up
Translation, ribosomal structure, and biosynthesis	50 S ribosomal protein L10	1	Down
	Elongation factor G	15	Disappeared
	Elongation factor P	16	Disappeared
	Elongation factor TU	18, 19	Down
	Polypeptide deformylase	51	Down
Membrane proton transport	Putative transport system permease	58	Down
	Calcium-binding protein ^a	4	Up
Signal transduction	Glyceraldehyde 3-phosphate dehydrogenase ^a	26	Down
	NADP-dependent GAPDH ^a	45	Down
	Phosphotransacetylase	50	Down
	Protein-tyrosine phosphatase	32	Up
Virulence factor and cell wall adhesion	Tetravalent M protein	73	Disappeared
	M5 protein	74	Disappeared
	Cysteine protease SpeB	69–72	Up
	dTDP-4-keto-6-deoxyglucose-3,5-epimerase ^a	40	Down
	Superoxide dismutase ^a	64	Down
	Alkyl hydroperoxidase ^a	38	Down
	Calcium-binding protein ^a	4	Up
GAPDH ^a	26	Down	
Cell wall synthesis	dTDP-4-keto-6-deoxyglucose-3,5-epimerase ^a	40	Down
Unknown	YomF	66	Down
	Subtylisin Dy	63	Up

^a Proteins that have multiple functions in the bacteria.

the induction of general stress proteins and a variety of other proteins essential to the stress response. The SigmaB regulon has been well-characterized in many bacteria, but is incompletely understood in *Streptococcus* (33, 34). We postulate that increased expression of RsbU represents an attempt by *S. pyogenes* to activate the SigmaB stress response to cope with the effects of fluoride.

Several examples are present in the literature of the application of proteomic analysis to the study of *Streptococcus* species (19, 35, 36). None of these studies examined the effect of fluoride on *S. pyogenes* and cannot be used to confirm our

findings. Many previous studies have determined that fluoride inhibits the function of several proteins, including catalase, superoxide dismutase, and elongation factor G (37, 38). These studies did not examine the effect of fluoride on the amount expressed of these proteins. Our data indicate that effect of fluoride on the function of many proteins may result from decreased expression as well as the previously observed inhibition of protein function.

Our proteomic analysis also suggests a new hypothesis to test regarding the effect of fluoride on *S. pyogenes*. We observed that fluoride altered the expression of proteins essential to the

non-suppurative complications of *S. pyogenes* infection, including rheumatic fever and APSGN. We observed decreased expression of GAPDH, GroEL, and DnaK proteins previously implicated in the pathogenesis of ASPGN and rheumatic fever (39–42). We further observed a decrease in the amount of M protein released into the supernatant by fluoride-treated bacteria. M protein has been implicated in the pathogenesis of both rheumatic fever and ASPGN. One putative mechanism for M protein's role in these disorders is presumably the cross-reaction of anti-M protein antibodies with host myosin proteins (43, 44).

Several factors may have contributed to the decreased incidence of rheumatic fever and APSGN in industrialized countries, including the introduction of antibiotics, aggressive treatment of streptococcal pharyngitis, and improved public health measures. However, the decline in rheumatic fever and ASPGN also began at the time that fluoridation of water supplies was introduced (45–47). Our data suggest the hypothesis fluoridation of water may have influenced the decline in non-suppurative *S. pyogenes* complications.

In summary, we have used proteomic analysis to construct a reference proteome map for *S. pyogenes*. This map can then be used to study a large number of proteins simultaneously from any interventions. Several cellular and extracellular proteins were altered by fluoride. We postulate that fluoride may affect defense mechanisms, virulence, and immunogenicity of the streptococci and may aid to a reduction of poststreptococcal sequelae. Further studies are needed to explore these complex mechanisms of fluoride on *S. pyogenes*.

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