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Comparative Proteomic Analysis of the *Haemophilus ducreyi* Porin-Deficient Mutant 35000HP::P2AB▽

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Haemophilus ducreyi **is an obligate human pathogen and the causative agent of the sexually transmitted, genital ulcerative disease chancroid. The genome of strain 35000HP contains two known porin proteins, OmpP2A and OmpP2B. Loss of OmpP2A and OmpP2B expression in the mutant 35000HP::P2AB resulted in no obvious growth defect or phenotype. Comparison of outer membrane profiles indicated increased expression of the 58.5-kDa chaperone, GroEL, in the porin-deficient mutant. A proteomics-based comparison resulted in the identification of 231 proteins present in membrane-associated protein samples, of which a subset of 56 proteins was differentially expressed at a level of 1.5-fold or greater in the porin-deficient strain 35000HP::P2AB relative to that in 35000HP. Twenty of the differentially expressed proteins were selected for real-time PCR, resulting in the validation of 90% of the selected subgroup. Proteins identified in these studies suggested a decreased membrane stability phenotype, which was verified by disk diffusion assay. Loss of OmpP2A and OmpP2B resulted in global protein expression changes which appear to compensate for the absence of porin expression in 35000HP::P2AB.**

Genital ulcers can result from infections with a number of sexually transmitted pathogens, including *Haemophilus ducreyi* (22). Infection with *H. ducreyi* is uncommon in the United States but has been identified as a cofactor in the transmission of human immunodeficiency virus in developing nations, where both diseases are endemic (14, 50). As with all gram-negative bacteria, the outer membrane (OM) is the primary permeability barrier for *H. ducreyi* (34, 35). Porin proteins are important components of the OM, comprising a significant portion of the OM protein content of and functioning as the primary means for hydrophilic solutes, wastes, and antimicrobial agents to cross the OM (34, 35). The genomes of enteric, gram-negative bacteria commonly possess several porin encoding genes (4, 19, 27, 37). However, the genome of 35000HP contains only two known porin genes, *ompP2A* and *ompP2B*. Interestingly, unlike 35000HP, most clinical isolates of *H. ducreyi* express OmpP2A exclusively (40). OmpP2A and OmpP2B share 27% to 33% homology with the OmpP2 porin of *Haemophilus influenzae* Rd (40, 45, 49). Deletion of *ompP2* in *H. influenzae* type b resulted in a construct with a pronounced growth defect that was avirulent in vivo (9). In contrast to results of these previous studies, the deletion of both *ompP2A* and *ompP2B* in 35000HP::P2AB had no statistically significant effect on pustule formation in the human challenge model (20).

In the present study, we performed a proteomics-based, comparative analysis of 35000HP::P2AB to 35000HP in order to identify protein expression differences that may correlate with phenotypic differences caused by (or resulting from) the absence of OmpP2A and OmpP2B. We have detected the expression of 231 proteins, a subset of which is differentially expressed at both the protein and transcript level. These results suggest that a global change in protein expression occurs in 35000HP::P2AB which functionally compensates for the loss of OmpP2A and OmpP2B.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. *H. ducreyi* strains 35000HP and 35000HP::P2AB have been described previously (20, 47). These strains were routinely cultured at 35.5°C on supplemented chocolate agar or in *H. ducreyi* broth as described previously (8).

Membrane-associated protein isolation and analysis. Total membrane preparations (MP) for sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) were isolated as previously described (46). MP samples for the Protein Biomarker Discovery Service (ProtTech Inc., Norristown, PA) were isolated as described previously (46), with the following modification: protein samples were resuspended in a modified buffer Z containing 50 mM HEPES (final concentration) substituted for 50 mM Tris, pH 8.0 (final concentration), to prevent interference with the lysine residue acylation reaction. SDS-PAGE and Western immunoblot analysis were performed as described previously (25). All lanes contained 10 μg/ml of protein as determined by the Lowry protein assay (Sigma-Aldrich, Springfield, MO).

Antibody development and characterization. We previously developed antisera specific to either OmpP2A or OmpP2B, and the development of monoclonal antibody (MAb) 2C7 has been described elsewhere (47). MAb 1B2-1B7 was purchased from the American Type Culture Collection and has been previously demonstrated to bind the lipooligosaccharide (LOS) of *H. ducreyi* (12, 13, 32, 54). The GroEL-specific MAb 2G3 was generated following whole-cell immunization with *H. ducreyi* strains 35000, CIP542, and 33921 utilizing a previously described protocol (7, 17).

RNA isolation. Broth cultures inoculated with either 35000HP or 35000HP::P2AB were grown to an optical density at 600 nm of 0.950. Ten-milliliter aliquots were immediately treated with RNAlater (Ambion, Austin, TX) to prevent RNA degradation. RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA samples were treated with Baseline-ZERO DNase (Epicentre, Madison, WI) to remove contaminating genomic DNA, and RNA clean up was performed using the RNeasy mini kit (Qiagen, Valencia, CA) RNA clean-up protocol as per the manufacturer's instructions. RNA was converted to cDNA using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA).

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TABLE 1. Nucleotide sequences of qRT-PCR primers used in this study

Primer	Sequence 5′ → 3′
aceE-F	ACCGAGCCTTCTTTCGCTAGTTGT
aceE-R	TACGGATGGCTTTGGTCGTTCTGA
argH-F	GCACAAATTAGTGGCAACGGCTGA
argH-R	GCCAATGCACCACAAAATGGA
cafA-F	AAACCGCCGSGTTCATCACAATGG
cafA-R	GAACCAAAGGCGCTCGCTTAACAA
frdA-F	TTAATGACGGAAGGGTGTCGTGGT
frdA-R	TTTGTCACGTGGGCCTAACTCCAT
groEL-F	AACTTTAGTGGTTAATACTATGCGTGGT
groEL-R	ACGGTCGCCGAAGCC
HD1190-F	AGTGCAACGGACGGTGGTAATACT
HD1190-R	GCTCAAGAAGAAGCAGGCGGTTTA
HD1337-F	GGTGCTTGTTTATGGGCTGCCATT
HD1337-R	CGAAGAAATTCGCGCGATTATTGCACA
HD1400-F	GCACTCTGGCAACAACAGCCATTA
HD1400-R	CACGCTCTTTGCTTAACGCTGTGA
HD1654-F	GCCATTTAATGTAACCGCAGGGCA
HD1654-R	TAAATGGCGCCAATCGGCATTACC
imp-F	ACTTTGCGGGCGAAGAAATTA
imp-R	TGTGGTGCCTGTTGTTCAATTCGG
mukB-F	GTTGTCTTGCTGTTGGCGGTGTA
mukB-R	CGGAATTTAATCAGCAAGCGGCGA
nudH-F	ATGACGCAAGTCAGCCGGTATGTA
nudH-R	AACCCAACGCCAACCATCAAACTC
nusG-F	TTACGATGGCGAGGTTTATCCGCA
nusG-R	TGCCACGTGTAATGGGCTTCATTG
putP-F	AGGCGGTCGTCGTTTAGGTAGTTT
putP-R	GCCCGCAACCAGTTTCCAGTTAAA
recB-F	GACTTTCAACGCGCAGCAGAACAT
recB-R	TTGCTTGGAATGGCTGAATAGCGG
rluB-F	ACACTTACTTTAGCCGGTCGGGTT
rluB-R	AATTCAGCACCGTTTAGCACACCC
rpoD-F	AGTACAGTGTGCGGTGGCTGAATA
rpoD-R	TCTGCGACCACATCTGATGCTTCT
secB-F	TAGAAGACAGTGGCGATACGGCAT
secB-R	AGTACGCTAGGGCATTGTGATGCT
suhB-F	TTTACTGCGGTACGTGGTGAAGGT
suhB-R	TAGCACCGGTTAAATCACGACGCT
uup-F	TTGCCGAACCTTGTAATTCACGCC
uup-R	AAGGTATTAAAGCACGCCGAACGC

RT-PCR analysis. All primers used in this study are listed in Table 1 and were designed using PrimerQuest Software (http://www.idtdna.com/Scitools/Applications/Primerquest/). Primer specificity and amplification efficiency were validated as described previously (29). Quantitative, real-time PCR (qRT-PCR) was performed in a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) RT instrument using the QuantiTect SYBR green PCR kit (Qiagen) using the following thermocycling parameters: 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. RT data were collected by the Rotor-Gene 6 analysis program and transformed using the $2^{-\Delta\Delta CT}$ method in Microsoft Excel 2007 prior to statistical analysis in GraphPad Prism 4.0 (La Jolla, CA) (29).

Disk diffusion assays. Disk diffusion assays were performed as described previously (30), with the following modifications. Chocolate agar plates, inoculated with 200 μl of 35000HP or 35000HP::P2AB suspended in brain heart infusion to an optical density at 600 nm of 0.2, were incubated for 30 min at 35.5°C, 5% CO_2 prior to application of paper disks saturated with the appropriate detergent or hydrophobic antibiotic. Each detergent or hydrophobic antibiotic was assayed in quadruplicate during three independent experiments at the following concentrations: cetyltrimethylammonium bromide (CTAB; 100 mg/ml), *N*-lauroyl sarcosine (100 mg/ml), SDS (100 mg/ml), Triton X-100 (10%, vol/vol), Tween 20 (10%, vol/vol), novobiocin (10 mg/ml), polymyxin B (10 mg/ml), and deoxycholate (100 mg/ml). All chemicals were purchased from Sigma-Aldrich (Springfield, MO). Statistical significance was determined by a paired, two-tailed Student's *t* test in GraphPad Prism 4.0.

Protein differential expression analysis. Differential expression of membrane-associated proteins was determined by the Protein Biomarker Discovery Service offered by ProtTech, Inc. This service utilizes 1-D gel electrophoresis coupled with subsequent light chromatography-tandem mass spectrometry (LC-MS-MS) analysis of isotope-coded affinity-tagged membrane-associated protein samples (28). This isotope-coded affinity-tagged technique, known as lysine-residue isotope tagging, is a proprietary extension of previously established N-terminal protein labeling techniques (33, 55, 56). Data analysis was performed as described previously (21).

MS analysis. Matrix-assisted laser desorption ionization-MS services were performed at the Department of Biochemistry Proteomics Core Facility, University at Buffalo, SUNY.

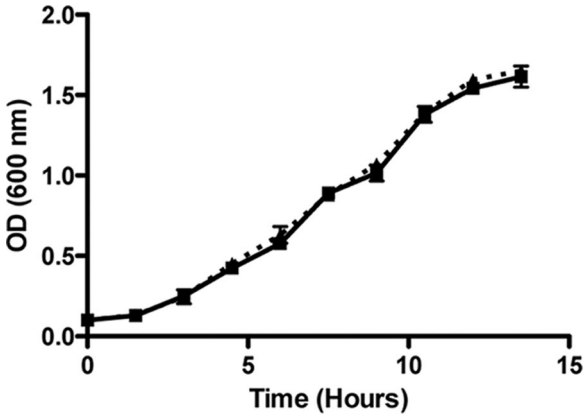

FIG. 1. Growth comparison of *H. ducreyi* 35000HP to the porin-deficient 35000HP::P2AB. The growth of 35000HP (squares) was compared to that of 35000HP::P2AB (triangles) in *H. ducreyi* broth. No discernible, statistically significant difference was identified in the doubling time of 35000HP compared to that of 35000HP::P2AB ($P = 0.482$; $n = 3$).

RESULTS

Characterization of the 35000HP::P2AB mutant. We have previously described the construction of a 35000HP mutant defective in expression of both OmpP2A and OmpP2B (20). Comparative growth analysis of 35000HP and 35000HP::P2AB demonstrated that the loss of both porins had no effect on growth in standard culture medium (Fig. 1). These data are in

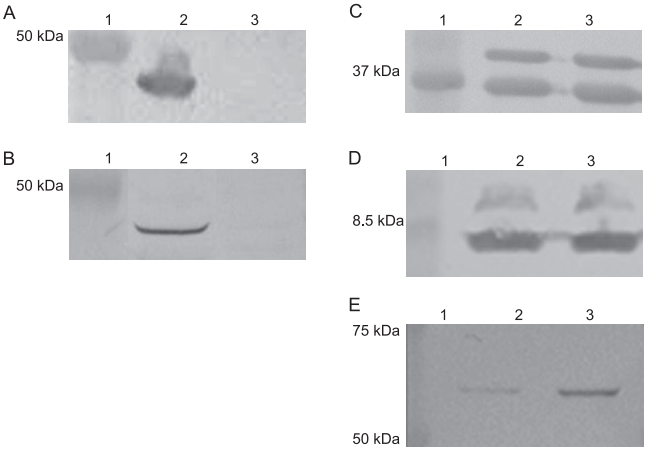

FIG. 2. Observation of the increased expression of a 58.5-kDa protein in the porin-deficient mutant. Western blot analysis of 35000HP (lane 2) and 35000HP::P2AB (lane 3) total MPs using antibodies specific to OmpP2A (A), OmpP2B (B), OmpA2/MOMP (C), LOS (D), and GroEL (E). Molecular size standards (lane 1) are shown in kilodaltons.

TABLE 2. Complete list of membrane-associated proteins identified by Protein Biomarker Discovery Service

NCBI RefSeq accession no.	Protein characteristic(s)[a]
NP_872672.1	Major OM protein homolog, OmpA2
NP_873123.1	Lipoprotein Hlp
NP_872671.1	Major OM protein
NP_872802.1	Periplasmic zinc transporter
NP_873651.1	Hypothetical OM protein
NP_873192.1	30S ribosomal protein S7[b]
NP_872695.1	Periplasmic nitrate reductase[b]
NP_873124.1	Lipoprotein HlpB[b]
NP_874240.1	FKBP-type peptidyl-prolyl *cis-trans* isomerase FkpA
NP_874283.1	30S ribosomal protein S4[b]
NP_872858.1	15-kDa OM lipoprotein[b]
NP_872909.1	Manganese superoxide dismutase
NP_874147.1	60-kDa chaperonin
NP_873267.1	Endo-1,4-beta-xylanase A[b]
NP_873991.1	30S ribosomal protein S2
NP_873158.1	Heme binding protein
NP_873136.1	Hypothetical protein HD0595[b]
NP_872850.1	Hypothetical protein HD0256
NP_874290.1	30S ribosomal protein S5[b]
NP_873316.1	Hypothetical protein HD0805[b]
NP_873652.1	OM protein D-15
NP_874221.1	50S ribosomal protein L7/L12
NP_874308.1	50S ribosomal protein L2[b]
NP_873552.1	Spermidine/putrescine-binding periplasmic protein
NP_874222.1	50S ribosomal protein L10
NP_873987.1	Ribosome releasing factor
NP_874254.1	Collagen adhesin NcaA
NP_874343.1	Hemoglobin-binding protein HgbA
NP_873635.1	OM protein P4
NP_872793.1	Chaperone protein DnaK
NP_874312.1	30S ribosomal protein S10[b]
NP_873494.1	DNA-binding protein HU[b]
NP_873733.1	Tight adherence protein D[b]
NP_874137.1	18,000-molecular-weight peptidoglycan-associated OM lipoprotein PAL
NP_873827.1	Hypothetical protein HD1409[b]
NP_874224.1	50S ribosomal protein L11[b]
NP_873538.1	Nucleoside diphosphate kinase
NP_874163.1	Translation initiation factor IF3[b]
NP_873902.1	30S ribosomal protein S9
NP_873019.1	Pyruvate kinase II
NP_873133.1	Hypothetical protein HD0591[b]
NP_873623.1	Large supernatant protein 2[b]
NP_873911.1	Large supernatant protein 1[b]
NP_874292.1	50S ribosomal protein L6[b]
NP_872899.1	Transaldolase
NP_874286.1	50S ribosomal protein L36[b]
NP_873852.1	OM protein P2 homolog
NP_874288.1	50S ribosomal protein L15[b]
NP_872690.1	Fine tangled pili major subunit
NP_872796.1	Hypothetical protein HD0192[b]
NP_874256.1	DNA-directed RNA polymerase omega subunit[b]
NP_873235.1	3-Oxoacyl-(acyl-carrier-protein) reductase, truncated
NP_872807.1	50S ribosomal protein L27[b]
NP_873584.1	Dihydrodipicolinate synthase[b]
NP_874160.1	Hypothetical protein HD1798[b]
NP_873288.1	50S ribosomal protein L32[b]
NP_873671.1	Hypothetical protein HD1218[b]
NP_872936.1	Heat shock protein HtpX[b]
NP_873637.1	RNA polymerase sigma-70 factor[b]
NP_873668.1	Small protein A[b]
NP_873029.1	Hypothetical protein HD0457[b]
NP_873262.1	RNA-binding protein Hfq[b]
NP_874092.1	50S ribosomal protein L31[b]
NP_874225.1	Transcription antitermination protein NusG
NP_874223.1	50S ribosomal protein L1
NP_873210.1	Hypothetical protein HD0680
NP_874284.1	30S ribosomal protein S11[b]
NP_872663.1	Coproporphyrinogen III oxidase[b]
NP_872680.1	Elongation factor Tu
NP_873194.1	Elongation factor Tu
NP_874042.1	Phosphoglyceromutase
NP_874291.1	50S ribosomal protein L18[b]
NP_874280.1	30S ribosomal protein S16[b]
NP_872641.1	ATP synthase subunit B[b]
NP_873551.1	HSP-70 cofactor
NP_873350.1	Superoxide dismutase [Cu-Zn][b]
NP_873801.1	Hypothetical protein HD1377[b]
NP_872827.1	Phosphotransferase system phosphocarrier protein HPr[b]
NP_873040.1	Conserved putative lipoprotein[b]
NP_873191.1	30S ribosomal protein S12[b]
NP_873285.1	Serum resistance protein DsrA
NP_873532.1	30S ribosomal protein S18[b]
NP_873221.1	RNA polymerase sigma factor[b]
NP_874075.1	Organic solvent tolerance protein precursor[b]
NP_873197.1	Export protein SecB
NP_873277.1	Lipoprotein[b]
NP_873102.1	Aminopeptidase A/I[b]
NP_873292.1	Hypothetical protein HD0776[b]
NP_874344.1	Large-conductance mechanosensitive channel[b]
NP_872854.1	Periplasmic serine protease do
NP_874177.1	Iron (chelated) ABC transporter, periplasmic-binding protein
NP_873279.1	Acid phosphatase stationary-phase survival protein[b]
NP_874108.1	Putative uroporphyrinogen III *C*-methyltransferase[b]
NP_872940.1	Hypothetical protein HD0358[b]
NP_874148.1	Co-chaperonin GroES
NP_874289.1	50S ribosomal protein L30[b]
NP_874140.1	Colicin transport protein, TolQ[b]
NP_874120.1	Ferritin-like protein 1
NP_874012.1	Dihydrolipoamide acetyltransferase
NP_873182.1	Hypothetical protein HD0646[b]
NP_873496.1	Hypothetical protein HD1006[b]
NP_873530.1	30S ribosomal protein S6
NP_874117.1	Preprotein translocase subunit YajC[b]
NP_874285.1	30S ribosomal protein S13[b]
NP_872698.1	Possible cytochrome *c*-type protein[b]
NP_872785.1	3-Hydroxydecanoyl-acyl carrier protein dehydratase[b]
NP_873254.1	50S ribosomal protein L28[b]
NP_873904.1	Hypothetical protein HD1495[b]
NP_874263.1	Acyl carrier protein
NP_873249.1	Hypothetical protein HD0725[b]
NP_874191.1	Xanthine phosphoribosyltransferase[b]
NP_873669.1	Hypothetical protein HD1215[b]
NP_873895.1	Integration host factor, alpha chain[b]
NP_874296.1	50S ribosomal protein L24[b]
NP_873876.1	Translation initiation factor IF2[b]
NP_872806.1	50S ribosomal protein L21[b]
NP_873499.1	Putative solute/DNA competence effector[b]
NP_874282.1	DNA-directed RNA polymerase alpha subunit
NP_874327.1	Hypothetical protein HD2003
NP_874111.1	ABC-type transport protein Uup[b]
NP_873974.1	Condensin subunit B
NP_874219.1	DNA-directed RNA polymerase beta subunit[b]
NP_873097.1	5″-Phosphoribosylglycinamide transformylase[b]
NP_873358.1	2-Dehydro-3-deoxyphosphooctonate aldolase[b]
NP_873559.1	Cysteine desulfurase
NP_873624.1	Anaerobic glycerol-3-phosphate dehydrogenase, subunit A[b]
NP_873795.1	Hypothetical protein HD1371[b]
NP_873981.1	ATP-dependent RNA helicase[b]
NP_874039.1	Hypothetical protein HD1654[b]
NP_874114.1	DNA gyrase subunit A[b]
NP_872657.1	Fumarate reductase
NP_872776.1	Dinucleoside polyphosphate hydrolase[b]
NP_872798.1	RNase E[b]
NP_873314.1	"*Haemophilus somnus*" lipoprotein C homolog[b]
NP_873489.1	Sodium/proline symporter, proline permease[b]
NP_873554.1	Exodeoxyribonuclease V, beta subunit[b]
NP_873994.1	GTP-binding protein Era[b]
NP_874019.1	Cytoplasmic axial filament protein[b]
NP_874161.1	50S ribosomal protein L20[b]
NP_874167.1	Thiamine biosynthesis protein ThiI[b]
NP_874218.1	DNA-directed RNA polymerase beta″ subunit[b]
NP_872924.1	Probable pseudouridylate synthase[b]
NP_873015.1	Inositol-1-monophosphatase[b]
NP_873352.1	DNA polymerase III subunit beta[b]
NP_873522.1	Argininosuccinate lyase[b]
NP_873683.1	DNA polymerase I[b]
NP_873822.1	Putative soluble lytic murein transglycosylase[b]

Continued on following page

TABLE 2—*Continued*

NCBI RefSeq accession no.	Protein characteristic(s)[a]
NP_873909.1	Inositol-5-monophosphate dehydrogenase
NP_873978.1	DNA topoisomerase IV subunit A[b]
NP_874013.1	2-Oxoglutarate dehydrogenase
NP_874067.1	Type III restriction enzyme[b]
NP_874255.1	ATP-dependent DNA helicase RecG[b]
NP_874330.1	ATP-dependent protease ATP-binding subunit[b]
NP_873678.1	Transcriptional regulatory protein[b]
NP_872770.1	RNase activity regulator protein RraA[b]
NP_873187.1	Opacity associated protein A[b]
NP_873533.1	50S ribosomal protein L9
NP_874341.1	Hypothetical protein HD2023[b]
NP_872931.1	Nitrate reductase, cytochrome *c*-type protein[b]
NP_873712.1	Protein-export membrane protein[b]
NP_873322.1	Arginine ABC transporter, periplasmic-binding protein[b]
NP_874304.1	50S ribosomal protein L16[b]
NP_874305.1	30S ribosomal protein S3[b]
NP_873175.1	Possible cell division protein[b]
NP_873241.1	Twin arginine translocase protein A[b]
NP_873417.1	Hypothetical protein HD0923[b]
NP_873558.1	Hypothetical protein HD1080[b]
NP_873615.1	Serine transporter[b]
NP_874095.1	Na^+/H^+ antiporter protein[b]
NP_874189.1	Probable OM protein[b]
NP_873253.1	50S ribosomal protein L33[b]
NP_874227.1	Putative oligopeptide transporter[b]
NP_874302.1	30S ribosomal protein S17[b]
NP_872773.1	Inorganic pyrophosphatase
NP_873718.1	Single-strand DNA-binding protein
NP_873875.1	Ribosome-binding factor A[b]
NP_873990.1	Elongation factor Ts
NP_874138.1	Colicin tolerance protein[b]
NP_874162.1	50S ribosomal protein L35[b]
NP_872836.1	*S*-Adenosyl-methyltransferase[b]
NP_872825.1	Glucose-specific phosphotransferase system enzyme IIA component[b]
NP_872882.1	Hypothetical protein HD0292[b]
NP_873347.1	Putative secreted protease[b]
NP_873491.1	Hypothetical protein HD1001[b]
NP_873542.1	Hypothetical protein HD1060[b]
NP_873812.1	Molybdopterin converting factor subunit 2[b]
NP_874081.1	Hypothetical protein HD1710[b]
NP_874171.1	Thioredoxin
NP_872949.1	Ferric uptake regulation protein[b]
NP_873141.1	Hypothetical protein HD0600
NP_873397.1	Cytolethal distending toxin protein A[b]
NP_874145.1	Cytochrome *d* ubiquinol oxidase, subunit I[b]
NP_874229.1	Peptide deformylase[b]
NP_873284.1	Mannose-specific phosphotransferase IIAB component
NP_873537.1	Probable pseudouridine synthase[b]
NP_873649.1	(3*R*)-Hydroxymyristoyl-acyl carrier protein dehydratase[b]
NP_873684.1	Outer-membrane lipoprotein carrier protein precursor[b]
NP_873695.1	50S ribosomal protein L25[b]
NP_873769.1	Thiol/disulfide interchange protein
NP_873885.1	Hypothetical protein HD1472[b]
NP_874309.1	50S ribosomal protein L23[b]
NP_874277.1	50S ribosomal protein L19[b]
NP_872711.1	Hypothetical protein HD0097[b]
NP_872838.1	Penicillin-binding protein 3[b]
NP_872902.1	Dipeptide transport system permease protein[b]
NP_873167.1	2,3,4,5-Tetrahydropyridine-2-carboxylate *N*-succinyltransferase
NP_873199.1	Trigger factor
NP_873226.1	Possible TetR family transcriptional regulator[b]
NP_873335.1	UDP-glucose-4-epimerase[b]
NP_874007.1	Hypothetical protein HD1618[b]
NP_874027.1	Possible integral membrane protein of DedA family[b]
NP_874105.1	30S ribosomal protein S15[b]
NP_874192.1	Aminoacyl-histidine dipeptidase[b]
NP_874297.1	50S ribosomal protein L14[b]
NP_872715.1	Hypothetical protein HD0101[b]
NP_872816.1	Heme-binding protein A[b]
NP_872842.1	UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamate synthetase[b]
NP_872976.1	Electron transport complex protein RnfC
NP_873011.1	Transcription antitermination protein NusB[b]
NP_873283.1	Mannose-specific phosphotransferase system IIC component[b]
NP_873507.1	Probable macrolide-specific efflux protein[b]
NP_873672.1	Hypothetical protein HD1219[b]
NP_873685.1	Ribosomal protein S6 modification protein[b]
NP_873703.1	Exoribonuclease II[b]
NP_873728.1	Recombination factor protein RarA[b]
NP_873764.1	Hypothetical protein HD1332[b]
NP_873846.1	Alanyl-tRNA synthetase[b]
NP_873849.1	Hypothetical protein HD1432[b]
NP_873984.1	Pyridoxine biosynthesis protein[b]
NP_874196.1	TDP-4-keto-6-deoxy-D-glucose transaminase[b]
NP_874310.1	50S ribosomal protein L4[b]

[a] Proteins are listed in order of relative abundance from greatest to least.
[b] Proteins that have not been identified in previous *H. ducreyi* proteomics studies (39, 43).

striking contrast to previous studies describing a severe growth defect in an *H. influenzae* type b OmpP2 mutant (9). In addition, deletion of classical porins in other gram-negative bacteria often results in either a growth defect or a lethal phenotype (1, 6, 8–10, 41). To explore possible explanations for this unexpected result, MPs were isolated from 35000HP and 35000HP::P2AB and analyzed for any differences in the protein profiles by SDS-PAGE (data not shown) and Western blot analysis (Fig. 2). Western blots probed with OmpP2A-specific (Fig. 2A) and OmpP2B-specific (Fig. 2B) antisera confirmed the proper phenotypes of 35000HP and 35000::P2AB. Whereas Western blots probed with the OmpA homolog-specific MAb 2C7 (Fig. 2C) and LOS-specific MAb 1B2-1B7 (Fig. 2D) demonstrate that 35000HP and 35000::P2AB expressed equivalent levels of these membrane components, a Western blot probed with MAb 2G3 demonstrated increased reactivity to 35000HP::P2AB relative to 35000HP (Fig. 2E). MAb 2G3 reacts to a 58.5-kDa protein with an apparent molecular weight consistent with the *H. ducreyi* heat shock and chaperonin protein GroEL (unpublished results). To confirm that MAb 2G3 was specific to GroEL, the 58.5-kDa band was excised and subjected to matrix-assisted laser desorption ionization-MS and peptide mass fingerprint analysis. The NCBI database was queried using the Mascot search engine (http://www.matrixscience.com), which returned a single, high-probability hit (Mowse score, 160) to the *H. ducreyi* GroEL.

Comparative proteomic analysis. To determine if the increase in GroEL expression represented a singular response or was indicative of one or more previously unrecognized phenotypes, MP preparations from 35000HP and 35000HP::P2AB were compared for differential protein expression by ProtTech, Inc. (Norristown, PA). This 1-D gel electrophoresis coupled with a subsequent LC-MS-MS-based technique provides enhanced identification of membrane-associated and hydrophobic proteins compared to 2-D electrophoresis-based approaches (16, 52). A total of 231 proteins were identified between both strains, of which 170 (74%) have not been previously detected in prior *H. ducreyi* proteomics studies (Table 2), thus demonstrating the value of this method as a complement to standard 2-D electrophoresis-based techniques (39,

TABLE 3. Differential expression of membrane-associated proteins in 35000HP::P2AB relative to that in 35000HP

NCBI RefSeq accession no.	Protein	Function	COG assignment	No. of peptides[a]	Fold change in expression[b]
NP_872657.1	FrdA	Fumarate reductase (flavoprotein subunit)	C	2	4.2
NP_872698.1	NapB	Nitrate reductase (cytochrome *c* subunit)	C	4	1.7
NP_872776.1	NudH	Dinucleoside polyphosphate hydrolase	LR	2	2.2
NP_872819.1	ClpX	Periplasmic protease	O	4	ND
NP_872924.1	RluB	Probable pseudouridylate synthase	J	2	33.3
NP_872976.1	RnfC	Iron-sulfur binding NADH dehydrogenase	C	5	ND
NP_873015.1	SuhB	Inositol-1-monophosphatase	G	2	5.9
NP_873097.1	PurT	Phosphoribosylglycinamide formyltransferase	F	4	ND
NP_873171.1	AccC	Acetyl coenzyme A carboxylase (biotin carboxylase subunit)	I	3	ND
NP_873182.1	HD0646	Ligand-gated channel (CirA superfamily)	S	1	ND
NP_873191.1	RpsL	30S ribosomal protein S12	J	4	2.0
NP_873197.1	SecB	Chaperone/export protein	U	2	ND
NP_873221.1	RpoD	RNA polymerase general sigma factor	K	3	2.6
NP_873288.1	RpmF	50S ribosomal protein L32	J	2	ND
NP_873292.1	HD0776	Protein of unknown function	S	3	ND
NP_873489.1	PutP	Proline permease	ER	2	6.7
NP_873522.1	ArgH	Argininosuccinate lyase	E	2	3.9
NP_873554.1	RecB	Exodeoxyribonuclease V (beta subunit)	L	2	25.0
NP_873651.1	HD1190	Predicted OM protein (OmpH-like)	M	2	2.0
NP_873672.1	HD1219	Protein of unknown function	S	3	ND
NP_873728.1	RarA	DNA recombination factor	L	8	ND
NP_873766.1	Kgd	Alpha-ketoglutarate decarboxylase	C	4	ND
NP_873801.1	HD1377	Protein of unknown function	S	3	6.3
NP_873812.1	MoaE	Molybdopterin converting factor (subunit 2)	H	1	ND
NP_873822.1	HD1400	Putative soluble lytic murein transglycosylase	M	2	2.1
NP_873873.1	Pta	Phosphate acetyltransferase	CR	3	ND
NP_873912.1	ManB	Probable phosphomannomutase	G	4	ND
NP_873974.1	MukB	Chromatin remodeling	D	2	2.1
NP_873995.1	Rnc	RNase III	K	3	ND
NP_874012.1	AceF	Dihydrolipoamide acetyltransferase	C	11	ND
NP_874147.1	GroEL	60-kDa chaperonin	O	12	3.3
NP_874148.1	GroES	Co-chaperonin with GroEL	O	4	2.0
NP_874162.1	RpmI	50S ribosomal protein L35	J	3	3.3
NP_874219.1	RpoB	DNA-directed RNA polymerase (beta subunit)	K	2	ND
NP_874225.1	NusG	Transcription antitermination protein	K	2	3.6
NP_874227.1	HD1887	Putative oligopeptide transporter	S	1	ND
NP_873852.1	OmpP2B	Porin	M	1	ND*
NP_873623.1	LspA2	Hemagglutinin-like macrophage phagocytic inhibitor	U	30	−2.0
NP_874013.1	AceE	Pyruvate dehydrogenase	C	2	−1.9
NP_874019.1	CafA	RNase G	J	2	−9.4
NP_874039.1	HD1654	Protein of unknown function	S	2	−4.0
NP_874075.1	Imp	LPS/LOS export and organic solvent tolerance protein	M	3	−1.6
NP_874111.1	Uup	ABC-type transport protein	R	2	−1.5
NP_874280.1	RpsP	30S ribosomal protein S16	J	3	−1.5
NP_874286.1	RpmJ	50S ribosomal protein L36	J	8	−2.0
NP_873133.1	HD0591	Putative two-component sensor kinase of LemA family	S	3	−1.5
NP_873235.1	FabG	3-Oxoacyl-(acyl carrier protein) reductase	IQR	1	ND*
NP_873285.1	DsrA	Serum resistance protein	UW	1	ND*
NP_873911.1	LspA1	Hemagglutinin-like macrophage phagocytic inhibitor	U	10	−2.0
NP_874095.1	NhaB	Na^+/H^+ antiporter protein	P	1	ND*
NP_874191.1	Gpt	Xanthine-guanosine phosphoribosyltransferase	F	1	ND*
NP_874224.1	RplK	50S ribosomal protein L11	J	1	ND*
NP_874256.1	RpoZ	DNA-directed RNA polymerase omega subunit	K	4	−2.0
NP_874282.1	RpoA	DNA-directed RNA polymerase alpha subunit	K	4	−2.0
NP_874285.1	RpsM	30S ribosomal protein S13	J	2	−2.0
NP_874312.1	RpsJ	30S ribosomal protein S10	J	5	ND*

[a] Number of peptides used to identify the protein by LC-MS-MS analysis. The average differentially expressed protein was identified by 3.7 ± 0.6 peptides.
[b] Several proteins were present in detectable quantities in one but not both samples, preventing quantitative analysis of protein expression. ND, not detected in 35000HP; ND*, not detected in 35000HP::P2AB.

43). In total, 56 proteins were identified as being differentially expressed in the porin-deficient mutant relative to 35000HP (1.5-fold or greater), with the average differentially expressed protein being identified by 3.7 ± 0.6 peptides. Thirty-six proteins were increased in expression in 35000HP::P2AB relative to that in 35000HP, including the cytoplasmic chaperone SecB, the proline permease PutP, and the stress-associated dinucleoside polyphosphate hydrolase NudH. Twenty proteins were decreased in expression in 35000HP::P2AB relative to that in 35000HP, including the LOS/lipopolysaccharide (LPS) export

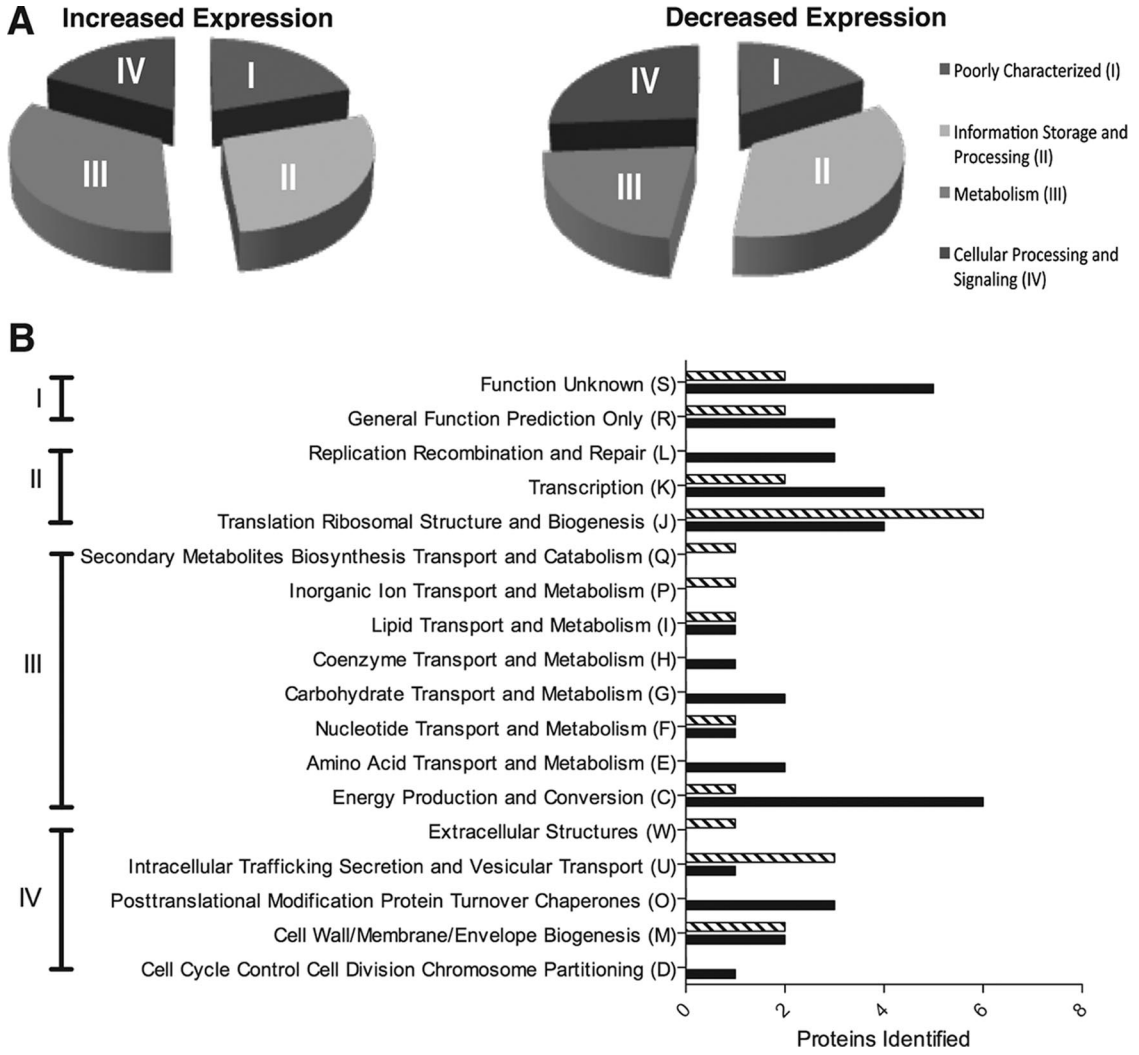

FIG. 3. COG classification of differentially expressed proteins in 35000HP::P2AB relative to those in 35000HP. (A) Proteins whose expression increased or decreased in 35000HP::P2AB relative to those in 35000HP were grouped by similar functional category: I, information storage and processing; II, cellular processing and signaling; III, metabolism; and IV, poorly categorized. (B) Differential expression of proteins within similar functional categories was further delineated by individual COG assignment. Proteins demonstrating increased expression in 35000HP::P2AB are denoted by solid bars, while proteins demonstrating decreased expression are denoted by hatched bars. Several proteins have multiple COG assignments; COG assignments for individual proteins are listed in Table 3.

protein Imp, the serum resistance protein DsrA, and the antiphagocytic proteins LspA1 and LspA2. A complete list of the differentially expressed proteins, exhibiting 1.5- to 33.3-fold changes in expression, is shown in Table 3. When the differentially expressed proteins were organized by the cluster of orthologous groups (COG) entry present for each protein in the 35000HP genome, a number of functional categories was identified (Fig. 3). Closer analysis of each functional category yielded interesting results (Fig. 3B). In particular, the number of differentially expressed metabolism-associated proteins indicated that a wide variety of metabolic processes had been affected. This change in metabolism is also mirrored by changes in proteins involved in transcription and translation. These data suggest that OmpP2A and OmpP2B may function as general diffusion pores, as has been described for OmpP2 of *H. influenzae* (51). Further analysis of the differentially expressed proteins identified a subset involved in membrane biogenesis. The number of affected chaperone, secretory, peptidoglycan, and membrane-associated proteins also suggests that OmpP2A and OmpP2B function in a structural capacity in the OM of 35000HP and that the loss of these proteins could result in a membrane biogenesis and/or stability defect. Finally, the loss of OmpP2A and OmpP2B resulted in the differential expression of several proteins with no defined function.

Verification of proteomics data by qRT-PCR. We validated our proteomics data by qRT-PCR analysis to correlate gene expression with protein expression. Twenty of the 56 proteins were selected for verification, representing slightly more than a third of the differentially expressed protein data set. These data correlated with the protein expression results, serving to validate 18 of the 20 selected proteins and representing a 90% accuracy rate for the Protein Biomarker Discovery Service (Table 4). The change in gene expression for *cafA* and *mukB* failed to meet the 1.5-fold cutoff value, indicating that either

TABLE 4. qRT-PCR analysis of gene expression for differentially expressed proteins

NCBI accession no.	Gene	Mean fold change ± SE[a]
gi33149018	*groEL*	4.0 ± 2.5
gi33151304	*frdA*	5.5 ± 1.0
gi33151423	*nudH*	2.0 ± 0.4
gi33151571	*rluB*	1.7 ± 0.06
gi33151662	*suhB*	2.2 ± 0.3
gi33151844	*secB*	2.1 ± 0.7
gi33151868	*rpoD*	1.9 ± 0.3
gi33152136	*putP*	2.1 ± 0.04
gi33152169	*argH*	2.3 ± 0.6
gi33152201	*recB*	3.4 ± 1.1
gi33152298	HD1190	4.1 ± 2.3
gi33152448	HD1377	2.6 ± 0.6
gi33152469	HD1400	1.9 ± 0.2
gi33152621	*mukB*	1.2 ± 0.09
gi33152660	*aceE*	−3.5 ± 0.2
gi33152666	*cafA*	−1.4 ± 0.1
gi33152686	HD1654	−2.3 ± 0.1
gi33152722	*imp*	−3.4 ± 0.8
gi33152758	*uup*	−5.5 ± 1.0
gi33152872	*nusG*	2.3 ± 0.5

[a] Mean fold change in gene expression in 35000HP::P2AB relative to 35000HP, normalized to *gyrA* expression as previously described (32).

their cognate proteins are not increased in expression in the porin-deficient mutant or that posttranscriptional regulatory mechanisms are responsible for the increase in expression detected by the Protein Biomarker Discovery Service.

35000HP::P2AB exhibits increased membrane permeability to hydrophobic agents. Changes in proteins associated with LPS/LOS export (Imp), peptidoglycan biosynthesis (HD1400), the OM (LspA1/2 and DsrA), and stress-associated chaperone function (GroEL/ES) in 35000HP::P2AB suggested that the loss of OmpP2A and OmpP2B may play a role in maintaining the structural integrity of the OM. We initially performed antibiotic sensitivity studies which showed that 35000HP::P2AB was more susceptible to erythromycin, a porin-independent antibiotic, but more resistant to the porin-dependent antibiotics tetracycline, ciprofloxacin, and tigecycline compared to the wild type (data not shown). We performed subsequent disk diffusion assays assessing the stability of the membrane to challenge from detergents and hydrophobic antibiotics. These data demonstrated that the porin-deficient mutant was more sensitive than the wild type to all detergents tested with the exception of the cationic detergent CTAB (Fig. 4). 35000HP::P2AB also exhibited increased sensitivity to hydrophobic antibiotics that do not enter the cell through porin proteins (Fig. 4 and data not shown). Taken together, these data indicate that the loss of OmpP2A and OmpP2B renders the OM more permeable to hydrophobic solutes.

DISCUSSION

The loss of classical porin expression in gram-negative pathogens often results in decreased fitness in both in vitro and in vivo environments (1, 6, 8–10, 41). However, 35000HP::P2AB did not exhibit any loss of viability or demonstrate any growth defect in vitro or in vivo in the human challenge model (20). Proteomic comparison of 35000HP::P2AB to 35000HP identified 231 proteins, 56 of which were determined to be differentially expressed. The differentially expressed proteins represented 18 separate COG classifications whose functions were predominantly associated with metabolism, protein trafficking, and membrane biogenesis. Differential expression was verified by qRT-PCR for 18 out of 20 selected proteins, representing a 90% success rate among the tested subset. Taken together, we suggest that the loss of OmpP2A and OmpP2B expression in 35000HP::P2AB results in global changes in protein expression and affects a wide range of cellular processes, the alteration of which appears to compensate for the loss of porin function in standard growth conditions.

Analysis of the COG assignments of the differentially expressed proteins indicates several interesting deviations in global protein expression in 35000HP::P2AB relative to that in 35000HP. Alterations in the metabolism-associated proteins are more numerous than any other COG category. Increased expression of the putative oligopeptide permease HD1887 and the proline permease PutP is anticipated to facilitate increased proline uptake in the porin-deficient mutant, an activity that has been demonstrated to help *Escherichia coli* survive a multitude of environmental stresses (23, 44, 53). Likewise, expression of argininosuccinate lyase (ArgH) was similarly increased. ArgH catalyzes the conversion of argininosuccinate into fumarate and arginine. Arginine stockpiling has also been demonstrated to occur in *E. coli* under a number of stressful growth conditions, including low pH and phosphate, nitrogen, and carbon deprivation (11, 18, 34). Such changes suggest that OmpP2A and OmpP2B function as general diffusion pores and/or facilitate the specific uptake of one or more cofactors involved in multiple metabolic pathways (2, 51).

The decreased expression of LspA2 and DsrA in the porin-deficient mutant is another interesting observation. Although the mechanisms that transport LspA2 and DsrA to the OM and surface are different, the energy expended in synthesizing

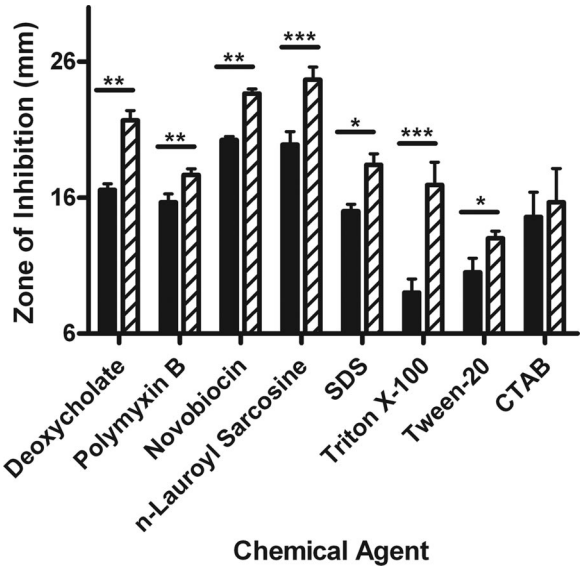

FIG. 4. Disk diffusion analysis of membrane stability. Disk diffusion assays were performed to evaluate the stability of 35000HP (solid bars) and 35000HP::P2AB (hatched bars). Asterisks denote values that differ in a statistically significant manner at a P value of 0.05 (*), 0.005 (**), or 0.0005 (***).

and exporting these proteins is fairly significant. Thus, it is possible that the decreased expression of these two proteins may represent an attempt to minimize the metabolic burden on the porin-deficient mutant consistent with the alterations in metabolic activity mentioned above. In contrast, expression of MOMP and OmpA2, two major OM protein components, was unchanged. These data suggest that these last two proteins are more important for the survival and growth of the porin mutant in vitro than LspA2 and DsrA. However, far more mechanistic studies are needed to more accurately address these observations.

GroEL involvement in stress responses is well studied for many bacteria, including *H. ducreyi* (18, 26, 36, 38, 48). 35000HP::P2AB exhibits increased expression of NudH, a dinucleoside polyphosphate hydrolase involved in the breakdown of toxic compounds, maintenance of normal metabolite pools, and the degradation of intercellular signaling molecules, including the alarmone diadenosine tetraphosphate (AP4A) (3, 31). As diadenosine oligophosphates such as AP4A have been demonstrated to link chaperone expression and stress responses in other organisms, it is possible a similar mechanism is at work in 35000HP::P2AB (3, 31). Similarly, NudH may function to integrate membrane and metabolic stress responses within the porin-deficient mutant (3, 31).

SecB is a cytoplasmic chaperone responsible for the primary binding of nascently synthesized polypeptides bound for the OM (11). Increased SecB expression in 35000HP::P2AB is matched by a similar increase in the expression of the conserved hypothetical protein, HD1190. In silico analysis of HD1190 demonstrated the presence of an OmpH-like sequence motif by CDART (conserved domain architecture tool) analysis and that it is highly similar to OmpH-like proteins of other *Pasteurellaceae* by BLAST (15). OmpH, also known as Skp, is one of three periplasmic chaperones that bind immature OMPs and target them to the OM for transport (24, 42). Several prominent secreted and OM proteins, notably LspA1/2 and DsrA, are decreased in expression in the porin-deficient mutant. The changes in translation-associated, chaperone, and OM protein expression in 35000HP::P2AB suggest alterations in protein export and/or secretion in the absence of OmpP2A and OmpP2B.

Increased expression of HD1400, a putative lytic murein transglycosylase may permit increased protein secretion into the periplasm or may increase cell wall permeability. The loss of OmpP2A and OmpP2B also resulted in the decreased expression of the Imp protein. In *E. coli*, Imp is an essential OM protein required for the export of LPS (5). Phenotypic analyses of Imp mutants demonstrated increased sensitivity to detergents and hydrophobic antibiotics, as well as increased membrane permeability to maltodextrins (5, 41). As inferred from the decreased expression of Imp in 35000HP::P2AB, the porin-deficient mutant exhibits increased sensitivity to both hydrophobic antibiotics and detergents (Fig. 4). These observations suggest that 35000HP::P2AB may be subject to increased cell envelope permeability, at both the OM and the cell wall, and that this property may partially offset the loss of OmpP2A and OmpP2B in a nonspecific manner. The decreased membrane stability of the porin-deficient mutant is interesting because this strain remains virulent in the human challenge model (20). It is possible that clearance of *H. ducreyi* in this model does not involve membrane stability and it is also possible that the porin-deficient mutant may be less virulent in the later stages of infection, a parameter that cannot be measured in this human system. However, more studies are needed to address these hypotheses.

To our knowledge, this report constitutes the first comparative proteomic analysis of a bacterium deficient in the expression of both known porin proteins. While the loss of OmpP2A and OmpP2B expression in 35000HP::P2AB has multiple effects on bacterial physiology, this mutant has no obvious growth defect and remains fully virulent in vivo (20). The survival of the porin-deficient mutant is an important observation as porin mutants in *Haemophilus* spp. exhibit severe phenotypic defects, including loss of viability (9). Additionally, we cannot rule out the possibility that other presently undetected proteins may also demonstrate altered expression or function to compensate for the loss of OmpP2A and OmpP2B in 35000HP::P2AB. We are currently extending our analyses of the proteins identified in this study with particular emphasis on membrane stability and permeability, metabolic profiling, and nutrient uptake. These data will be instrumental in characterizing the general and specific functions of OmpP2A and OmpP2B for *H. ducreyi* biology and pathogenesis.

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