Antibiotic resistance and expression of resistance-nodulation-division pump- and outer membrane porin-encoding genes in *Acinetobacter* species isolated from Canadian hospitals

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BACKGROUND: Bacterial pathogens belonging to the genus *Acinetobacter* cause serious infections in immunocompromised individuals that are very difficult to treat due to their extremely high resistance to many antibiotics.
OBJECTIVE: To investigate the role of resistance-nodulation-division (RND) pumps and porins in the antibiotic resistance of *Acinetobacter* species collected from Canadian hospitals.
METHODS: Clinical isolates of *Acinetobacter* species collected from Canadian hospitals were analyzed for the expression of genes encoding RND pumps (*adeB*, *adeG*, *adeJ*, *AciBau_2746* and *AciBau_2436*) and outer membrane porins (*carO*, 33 kDa porin and *oprD*) using quantitative reverse transcription (qRT) polymerase chain reaction. Species identification of the isolates was performed using a multiplex polymerase chain reaction method for *gyrB*.
RESULTS: The expression of RND pump-encoding genes was widespread in the clinical isolates of *Acinetobacter* species, with each of the isolates expressing at least one RND pump. *adeG* was found to be overexpressed in all of the isolates, while *adeB* was found to be overexpressed in only two isolates. Among the porin-encoding genes, the expression of *carO* was considerably downregulated among the majority of isolates.
CONCLUSION: The present study was the first to analyze the expression of RND pump- and porin-encoding genes in the clinical isolates of *Acinetobacter* species from Canadian hospitals. The overexpression of genes encoding RND pumps and the downregulation of genes encoding porins was common in clinical isolates of *Acinetobacter* species from Canadian hospitals, with the AdeFGH pump being the most commonly expressed RND pump.

Key Words: *Acinetobacter*; *Antibiotic resistance; Gene expression*

L'antibiorésistance et l'expression des gènes codants des pompes d'efflux RND et des porines de la membrane externe des espèces d'*Acinetobacter* isolées dans des hôpitaux canadiens

HISTORIQUE : Les pathogènes bactériens qui appartiennent au gène *Acinetobacter* provoquent de graves infections chez les personnes immunocompromises. Ces infections sont très difficiles à traiter en raison de leur résistance extrêmement élevée à de nombreux antibiotiques.
OBJECTIF : Examiner le rôle des pompes RND (*resistance-nodulation-division*) et des porines dans l'antibiorésistance des espèces d'*Acinetobacter* prélevées dans des hôpitaux canadiens.
MÉTHODOLOGIE : Les chercheurs ont analysé des isolats cliniques d'espèces d'*Acinetobacter* prélevés dans des hôpitaux canadiens afin de déceler l'expression des gènes codants des pompes RND (*adeB*, *adeG*, *adeJ*, *AciBau_2746* et *AciBau_2436*) et des porines de la membrane externe (*carO*, porine 33 kDa et *oprD*) à l'aide de la réaction en chaîne de la polymérase après transcription inverse quantitative (qRT). Ils ont identifié les espèces d'isolats à l'aide d'une méthode de réaction en chaîne de la polymérase multiplexe pour le *gyrB*.
RÉSULTATS : L'expression de gènes codants de la pompe RND était généralisée dans les isolats cliniques des espèces d'*Acinetobacter*, chaque isolat exprimant au moins une pompe RND. Les chercheurs ont observé une surexpression de l'*adeG* dans tous les isolats, mais la surexpression de l'*adeB* dans seulement deux isolats. Parmi les gènes codants des porines, l'expression du *carO* présentait une régulation négative marquée dans la majorité des isolats.
CONCLUSION : La présente étude était la première à analyser l'expression des gènes codants de la pompe d'efflux RND et des porines dans les isolats cliniques d'espèces d'*Acinetobacter* provenant d'hôpitaux canadiens. La surexpression des gènes codants des pompes RND et la régulation négative des gènes codants des porines était courante dans les isolats cliniques d'espèces d'*Acinetobacter* provenant d'hôpitaux canadiens, la pompe AdeFGH étant la pompe d'efflux RND la plus exprimée.

The *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex consists of *A calcoaceticus*, *A baumannii*, *Acinetobacter pittii* (formerly *Acinetobacter GS3*) and *Acinetobacter nosocomialis* (formerly *Acinetobacter 13TU*) species (1). This complex is notorious for causing serious infections in immunocompromised individuals. Although recent studies have shown that infections caused by *A pittii* and *A nosocomialis* may not be as uncommon as previously believed (2), *A baumannii*, responsible for a variety of infections, is still considered to be the most prominent species in this genera. These infections include ventilator-associated pneumonia, endocarditis, surgical-site infections, septicemia and urinary tract infections (3). The mortality rates associated with infections caused by *A baumannii* can be very high, with the mortality rate of pneumonia reported to be as high as 73% (4). Outbreaks in hospitals have been reported from various geographical areas (5), and the incidence of *A baumannii*-mediated infection has been increasing steadily over the past two decades (6).

The multidrug-resistant (MDR) nature of *A baumanniii* infections has proven to be very challenging for clinicians. The organism frequently exhibits resistance to many classes of antimicrobial drugs, including β-lactams (penicillins and cephalosporins), carbapenems,

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TABLE 1
Antibiotic susceptibilities of *Acinetobacter* species isolates

Strain	Source	AMK	CFZ	FEP	CRO	CIP	CLI	IPM	GEN	LVX	MEM	MXF	TZP	TGC	SXT
AB004	Winnipeg, MB	32	>128	128	32	2	2	0.25	32	1	2	0.5	64	2	0.12
AB005	Winnipeg, MB	<1	128	≤1	≤1	≤0.06	1	0.25	≤0.5	0.12	0.25	≤0.06	≤1	0.5	≤0.12
AB006	Vancouver, BC	2	>128	4	16	0.5	2	0.25	2	0.25	1	0.25	8	1	≤0.12
AB007	Vancouver, BC	2	>128	8	8	1	1	0.25	1	0.25	0.5	0.12	16	0.5	0.5
AB008	Hamilton, ON	2	>128	2	4	0.12	1	0.25	0.5	≤0.06	0.5	≤0.06	≤1	0.5	0.25
AB009	Hamilton, ON	2	>128	2	8	0.5	0.5	0.25	0.5	0.12	0.5	0.12	≤1	0.5	0.25
AB010	Vancouver, BC	2	>128	4	16	0.5	1	0.25	1	0.25	1	0.12	8	0.5	>8
AB011	Winnipeg, MB	32	>128	64	16	2	2	0.5	32	0.5	2	0.5	32	2	≤0.12
AB012	Victoria, BC	2	>128	2	16	0.12	1	0.12	0.5	0.12	0.5	≤0.06	4	0.25	≤0.12
AB013	Vancouver, BC	16	128	16	16	1	1	0.25	32	0.5	1	0.5	≤1	2	≤0.12
AB014	Montreal, QC	2	>128	8	16	0.5	0.25	0.25	≤0.25	0.25	1	0.12	16	0.5	0.25
AB027	Montreal, QC	>64	>128	>128	>256	>16	1	32	32	16	16	8	>512	4	>8
AB028	Winnipeg, MB	2	>28	32	64	>16	0.5	0.25	>32	16	2	8	128	0.5	8
AB029	Winnipeg, MB	≤1	128	16	2	2	0.5	0.25	≤0.5	1	0.5	0.5	≤1	2	≤0.12
AB030	Winnipeg, MB	64	>128	>64	>64	>16	0.5	>32	>32	16	>32	>16	>512	>16	>8
AB031	Toronto, ON	2	>128	4	16	0.25	0.5	0.25	<0.5	0.25	1	0.12	4	8	4
ATCC19606	ATCC	8	128	16	32	1	1	0.25	8	0.5	1	0.5	4	1	8

Data expressed as µg/mL. AMK Amikacin; ATCC American Type Culture Collection; BC British Columbia; CFZ Cefazolin; CIP Ciprofloxacin; CLI Colistin; CRO Ceftriaxone; FEP Cefepime; GEN Gentamicin; IPM Imipenem; LVX Levofloxacin; MB Manitoba; MEM Meropenem; MXF Moxifloxacin; ON Ontario; QC Quebec; SXT trimethoprim/sulfamethoxazole; TGC Tigecycline; TZP Piperacillin-tazobactam

aminoglycosides, fluoroquinolones and tetracyclines (7). Therefore, the emergence of MDR strains of *A baumannii* is becoming a major concern in hospital settings. Mechanisms of resistance include production of β-lactamase (8), the presence of aminoglycoside-modifying enzymes (9), target-site mutations (10), loss of outer membrane permeability (11) and multidrug efflux systems (12). The latter two, outer membrane permeability and multidrug efflux systems (particularly those belonging to the resistance-nodulation-division [RND] family), constitute the most important mechanisms of intrinsic resistance in *Acinetobacter* species.

Little is known about the mechanisms of antibiotic resistance in *A baumannii* from Canadian hospitals. The research objectives of this study were to analyze the expression of RND efflux pump- and outer membrane porin-encoding genes in clinical isolates of *Acinetobacter* species and to determine whether there is a correlation between the expression of these genes and the intrinsic antimicrobial resistance of this organism.

METHODS

Bacterial strains and growth conditions

Clinical isolates of *Acinetobacter* species used in the present study are listed in Table 1. Isolates, displaying varying antibiotic susceptibility profiles, were obtained from Canadian hospitals, specifically, from intensive care units from 2006 to 2009, inclusively, as part of the Canadian Hospital Ward Antibiotic Resistance Surveillance (CANWARD) study (www.can-r.ca). *A baumannii* ATCC 19606 was used as the control strain. Lysogeny broth (LB) medium (Biobasic, Canada) (37°C) was used for the culturing of bacterial strains.

Genomic DNA extraction

Genomic DNA extraction was performed, following the manufacturer's instructions, using a commercially available kit (DNeasy, Qiagen, Canada). DNA was stored at −20°C until used.

Genotyping of *Acinetobacter* species isolates

Genotyping of *Acinetobacter* species isolates was performed using up to 5 ng of genomic DNA according to the multiplex polymerase chain reaction (PCR) method for *gyrB* described in the study by Higgins et al (13).

Antibiotic susceptibility assays

Antibiotic susceptibility of *Acinetobacter* species isolates to antibiotics commonly used for the treatment (Table 1) of *Acinetobacter* species infections was determined using the microbroth dilution method, which has been previously described (14).

RNA extraction and complementary DNA (cDNA) synthesis

Overnight cultures grown in LB medium at 37°C with shaking (200 rpm) were subcultured (1:100) in fresh LB medium (without antibiotics) and incubated. Cells were harvested at an A600 of approximately 0.8 and were frozen as pellets (−80°C) to facilitate lysis. RNA extractions were performed using a commercially available kit (RNeasy, Qiagen) according to manufacturer's instructions. RNA samples were treated with DNAse I (Qiagen), according to manufacturer's instructions, to remove any genomic DNA carryover from RNA extraction. Synthesis of complementary DNA (cDNA) was performed using the GoScript Reverse Transcriptase kit (Promega, USA) using 1.2 µg of total RNA in a 20 µL reaction volume according to the manufacturer's instructions. To confirm the absence of genomic DNA contamination of RNA samples in the quantitative reverse transcription (qRT)-PCR described below, the minus-RT control reaction was performed by excluding the reverse transcriptase enzyme.

qRT-PCR

qRT-PCR reactions were performed using SsoFast Evagreen Supermix (BioRad, Canada) to study the expression of RND pump- and outer membrane porin-encoding genes. The RND pump-encoding genes analyzed included *adeB*, *adeG*, *adeJ*, *AciBau_2436* and *AciBau_2746*; the outer membrane porin-encoding genes analyzed included *carO*, *oprD* and the 33 kDa porin. All primers used for analysis (designed using Oligoperfect software, Invitrogen, Canada, www.invitrogen.com), except those used for *adeE* and the 33 kDa porin, have been described elsewhere (15). The primers used for *adeE* were adeE_RT_F (5'–gaaacagagcgggttggtaa–3') and adeE_RT_R (5'–tgcctgcgttatttctaccc–3'), and those used for the 33 kDa porin gene were 33KD_RT_F (5'–atccaaaacgaccaagatgc–3') and 33KD_RT_R (5'–caaaaccgattgccatgtta–3'). 16S ribosomal RNA (rRNA) was used as the housekeeping gene, while *A baumannii* ATCC 19606 was used as the reference strain. The efficiencies of primers were tested by pooling the cDNA from all isolates, serially diluting the pool 10-fold and then generating a standard curve. Reactions were performed in a total volume of 15 µL using 300 nM of each primer and 5 µL of the cDNA template (diluted

1:20 from the cDNA synthesis reaction described above). At least two independent samples were analyzed for each target gene and all reactions were performed in triplicate. A no-template control was used in all reactions. The 16S rRNA reaction was used to rule out genomic DNA contamination from the minus-RT control reaction.

RESULTS

Genotyping of *Acinetobacter* species isolates

Results of the multiplex PCR for *gyrB* used for the genotyping of the *Acinetobacter* species isolates are shown in Figure 1. This method identified two of 16 (13%) isolates as *A pittii* (*Acinetobacter* species AB006 and *Acinetobacter* species AB007), four of 16 (25%) as *A nosocomialis* (*Acinetobacter* species AB004, *Acinetobacter* species AB005, *Acinetobacter* species AB011 and *Acinetobacter* species AB013) and nine of 16 (56%) isolates were identified as *A baumannii*. One isolate, *Acinetobacter* AB012, could not be identified conclusively because the PCR products observed could have originated from either *A baumannii* or *A nosocomialis*. Multiple individual colonies of *Acinetobacter* AB012 were tested to rule out contamination, and the results consistently indicated (data not shown) that it was, in fact, a pure culture.

Antibiotic susceptibility assays

Results of the antibiotic susceptibility assays are summarized in Table 1. The susceptibility profiles of the *Acinetobacter* species isolates varied considerably from extensively susceptible to MDR (concomitantly resistant to ≥3 different drug classes). Some of the most resistant isolates in the present study were *A baumannii* (*A baumannii* AB027, *A baumannii* AB028 and *A baumannii* AB030), particularly with respect to resistance to aminoglycosides, cephalosporins, fluoroquinolones, carbapenems, piperacillin/tazobactam, tigecycline and trimethoprim/sulfamethoxazole. Among the non-*A baumannii* isolates, AB004 and AB011 (both identified as *A nosocomialis*) displayed resistance to aminoglycosides, cephalosporins and piperacillin/tazobactam.

Expression analysis of RND pump- and outer membrane porin-encoding genes

The expression analysis of RND pump- and outer membrane porin-encoding genes is summarized in Table 2. The expression analysis of outer membrane porin-encoding genes *carO*, the 33 kDa porin and *oprD* revealed that the expression of *carO* was either considerably lower than or similar to the *A baumannii* ATCC 19606 control, with the exception of *A baumannii* AB009 (16-fold higher expression), *A baumannii* AB028 and *A baumannii* AB030 (four-fold higher expression for both). Expression of the 33 kDa porin gene was found to be higher in seven isolates, with >10-fold higher expression seen in four isolates (*A baumannii* AB009, *A baumannii* AB027, *A baumannii* AB028 and *A baumannii* AB030). Six isolates (*A nosocomialis* AB004, *A pittii* AB006, *A pittii* AB007, *A baumannii* AB010, *A pittii* AB011, and *A nosocomialis* AB013) exhibited lower expression of *oprD* compared with the control strain *A baumannii* ATCC 19606. All other strains showed at least a two-fold higher expression of *oprD* compared with the *A baumannii* ATCC 19606 control. One exception was *A baumannii* AB030, which showed a similar level of expression of *oprD* as the control strain.

As for the RND efflux pump-encoding genes, *adeB* was overexpressed only by *A baumannii* AB030 (5.5-fold) and *A baumannii* AB031 (approximately two-fold), while *adeG* was overexpressed by all

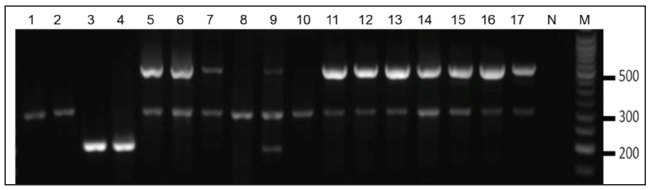

Figure 1) *Genotyping of* Acinetobacter *species isolates. Multiplex PCR for the* gyrB *gene identified clinical isolates as* Acinetobacter baumannii, Acinetobacter pittii *or* Acinetobacter nosocomialis. *Presence of a 294 bp band indicates* A nosocomialis, *and a 193 bp band indicates* A pittii. *Presence of two bands at 294 bp and 490 bp indicates* A baumannii. *Lane 1* Acinetobacter *species AB004, Lane 2* Acinetobacter *species AB005, Lane 3* Acinetobacter *species AB006, Lane 4* Acinetobacter *species AB007, Lane 5* Acinetobacter *species AB008, Lane 6* Acinetobacter *species AB009, Lane 7* Acinetobacter *species AB010, Lane 8* Acinetobacter *species AB011, Lane 9* Acinetobacter *species AB012, Lane 10* Acinetobacter *species AB013, Lane 11* Acinetobacter *species AB014, Lane 12* Acinetobacter *species AB027, Lane 13* Acinetobacter *species AB028, Lane 14* Acinetobacter *species AB029, Lane 15* Acinetobacter *species AB030, Lane 16* Acinetobacter *species AB031, Lane 17 A* baumannii *ATCC 19606. M Molecular weight marker; N No-template control*

TABLE 2
Expression* of resistance-nodulation-division pump† - and outer membrane porin-encoding genes in clinical isolates of *Acinetobacter* species

	Gene							
Isolate strain	***adeB***	***adeG***	***adeJ***	***AciBau_2746***	***AciBau_2436***	***carO***	***oprD***	**33kDa porin**
AB004	0.589±0.109	**4.452±0.81**	0.005±0.0005	0.004±0.001	0.006±0.003	0.003±0.0004	0.007±0.002	0.183±0.028
AB005	0.005±0.001	**6.271±0.81**	0.0002±0.000	0.155±0.017	0.051±0.007	0.003±0.0002	1.790±0.139	1.932±0.168
AB006	0.035±0.005	**3.339±0.337**	0.128±0.027	0.004±0.0020	0.012±0.002	0.002±0.0005	0.008±0.001	5.149±0.405
AB007	0.004±0.001	**3.594±0.357**	0.001±0.0004	0.247±0.026	0.003±0.0005	1.043±0.126	0.004±0.001	1.253±0.205
AB008	0.030±0.004	**8.658±0.662**	0.104±0.335	**4.167±0.304**	**2.401±0.157**	0.005±0.0004	5.636±0.451	0.492±0.024
AB009	0.004±0.001	**4.264±0.502**	**5.477±0.763**	0.009±0.005	0.020±0.003	16.328±2.171	3.513±0.436	12.855±2.431
AB010	0.013±0.004	**3.749±0.331**	0.232±0.299	0.007±0.001	0.007±0.002	0.008±0.001	0.008±0.001	7.439±0.606
AB011	1.256±0.179	**6.754±0.688**	0.255±0.017	0.007±0.002	0.019±0.009	0.005±0.001	0.008±0.001	0.324±0.023
AB012	0.027±0.021	**5.396±0.915**	**2.350±0.052**	0.002±0.0003	0.011±0.003	0.004±0.0004	2.016±0.083	8.480±1.926
AB013	0.804±0.243	**5.048±0.763**	0.038±0.043	0.008±0.005	0.008±0.001	0.004±0.0007	0.012±0.003	2.178±0.26
AB014	0.105±0.014	**8.803±1.356**	**3.916±0.269**	**3.684±0.178**	0.020±0.001	2.254±0.218	3.710±0.302	32.295±11.429
AB027	0.471±0.018	**2.122±0.206**	**3.721±0.155**	1.474±0.084	1.686±0.045	0.001±0.0002	2.971±0.114	23.381±3.238
AB028	0.108±0.006	**7.498±0.564**	**7.592±1.078**	**2.400±0.239**	0.020±0.003	4.051±0.502	4.160±0.275	0.387±0.045
AB029	0.037±0.007	**4.800±0.514**	0.691±0.047	**2.431±0.31**	0.013±0.004	0.225±0.022	3.426±0.301	0.223±0.017
AB030	**5.674±0.334**	**6.771±0.414**	**8.097±0.605**	1.904±0.121	0.086±0.011	4.485±0.326	1.397±0.095	21.052±1.256
AB031	1.876±0.17	**5.872±0.638**	**7.641±1.118**	0.852±0.601	0.019±0.002	0.151±0.023	4.822±0.464	1.897±0.349

*Data expressed as relative quantity ± SE. *Relative to the control strain ATCC 19606; †A >2-fold overexpression of resistance-nodulation-division efflux pump-encoding genes compared with the control strain ATCC 19606 is indicated in bold*

isolates. Seven isolates (*A baumannii* AB009, *Acinetobacter* species AB012, *A baumannii* AB014, *A baumannii* AB027, *A baumannii* AB028, *A baumannii* AB030 and *A baumannii* AB031) were found to overexpress *adeJ*. Among the two as yet uncharacterized RND pump encoding genes, *AciBau_2746* was overexpressed in six isolates while *AciBau_2436* was overexpressed in two.

DISCUSSION

In the past few decades, *A baumannii* has emerged as a major nosocomial pathogen capable of causing serious drug-resistant infections. The drug resistance of this organism poses a considerable challenge in the treatment of infections. In spite of the ever-increasing clinical importance of *Acinetobacter* species worldwide, little is known about the antibiotic resistance mechanisms used by these organisms. In the present study, we analyzed isolates of *Acinetobacter* species, collected from various Canadian hospital intensive care units, for expression of RND pump- and outer membrane porin-encoding genes.

RND pumps are believed to be the major determinants of intrinsic antibiotic resistance in Gram-negative pathogens and their overexpression is often linked to the MDR phenotype of these organisms. At present, three different RND pumps have been described in Gram-negative pathogens, namely, AdeABC (16), AdeIJK (17) and AdeFGH (18). AdeDE is another RND pump that has been reported in *A pittii* (19). In addition, a survey of the *A baumannii* ATCC 19606 genome revealed the presence of two more RND complex-encoding operons, namely AciBau_2434-5-6 and AciBau_2747-6.

In all 16 isolates, we analyzed the expression of three characterized and two as yet uncharacterized RND pump-encoding genes and compared their expression with that found in *A baumannii* ATCC 19606. We observed that all of the isolates expressed at least one RND pump-encoding gene. Previous studies have shown the AdeABC pump to be the major MDR pump in clinical isolates of Gram-negative pathogens (12,20), which is responsible for the efflux of amikacin, chloramphenicol, cefotaxime, erythromycin, gentamicin, kanamycin, norfloxacin, netilmicin, ofloxacin, pefloxacin, sparfloxacin, tetracycline, tobramycin and trimethoprim (16). In the present study, *A baumannii* AB030, an isolate found to be one of the most resistant, was among the two isolates exhibiting expression of *adeB* (Table 2). Interestingly, the levels of *adeB* in *A baumannii* AB027, another MDR isolate, were not found to be any different from any of the susceptible isolates (Table 1) suggesting that the expression of *adeB* is not solely responsible for the MDR phenotype of *A baumannii* AB030. However, we did observe a correlation between *adeB* expression and resistance to tigecycline. Both isolates, AB030 and AB031, which exhibited higher expression of *adeB*, were also found to be resistant to tigecycline. A similar correlation has also been reported previously (21).

AdeFGH and AdeIJK are two other pumps characterized from *A baumannii*. The AdeFGH pump is known to efflux chloramphenicol, trimethoprim, ciprofloxacin and clindamicin (18), while the AdeIJK pump effluxes β-lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampin, trimethoprim, acridine, pyronine, safranin and sodium dodecyl sulfate (17). We found *adeG* to be the most commonly expressed RND efflux pump gene among the isolates, followed by *adeJ*. *A baumannii* AB030, one of the most resistant isolates, was the only isolate that exhibited overexpression of *adeG* and *adeJ* in addition to *adeB*. We have previously shown the overexpression of *adeG* in some of these isolates using end-point RT-PCR (22), and the persent work further confirms that data using qRT-PCR. Three other isolates found to express three different RND pump-encoding genes, *A baumannii* AB008 (*adeG*, *AciBau_2436* and *AciBau_2746*), *A baumannii* AB014 (*adeG*, *adeJ* and *AciBau_2746*) and *A baumannii* AB028 (*adeG*, *adeJ* and *AciBau_2746*) did not exhibit susceptibilities different from isolates expressing fewer pumps. The lack of any obvious correlation between the expression of RND pump-encoding genes and resistance to their respective substrates tested in the present study was most likely due to the presence of other resistance mechanisms present in these isolates.

We also analyzed two as yet uncharacterized RND pump-encoding genes, namely, *AciBau_2436* and *AciBau_2746*. Sequence alignment indicated that these two genes likely encode metal efflux proteins and are not likely to play a role in the antibiotic resistance of *Acinetobacter* species isolates.

In addition, we analyzed the expression of *adeE* in two isolates identified as *A pittii* and found its expression to be nine-fold higher in *A pittii* AB007 compared with *A pittii* AB006 (data not shown). *A pittii* AB007 was also found to be moderately more resistant to ciprofloxacin, tazobactam, and trimethoprim/sulfamethoxazole (Table 1) than *A pittii* AB006, which could be due to the activity of the AdeDE pump.

Downregulation of outer membrane porins, sometimes with concurrent overexpression of RND pumps, is linked with antibiotic resistance in Gram-negative bacterial pathogens (23,24). All three porins analyzed in the present study have been implicated in carbapenem resistance in *Acinetobacter* species (25). Although we observed a considerable downregulation of *carO* in all but three isolates, namely, *A baumannii* AB009, *A baumannii* AB028 and *A baumannii* AB030 (Table 2), we did not notice any correlation with carbapenem resistance. Similarly, we did not observe any obvious correlations between *oprD* or 33 kDA porin gene expression and carbapenem resistance in the isolates (Tables 1 and 2). It is possible that the carbapenem resistance in the isolates was due to mutational alterations in the porin structures instead of changes in the expression levels (26). In addition, carbapenem resistance could also be a result of carbapenemase activity in the resistant isolates, the production of which was not analyzed in the present study.

Although the approach used in the present study only reveals RND pump-encoding genes that are transcribed constitutively due to regulatory mutations or the experimental conditions used, to our knowledge, it is the first to analyze the simultaneous expression of all known RND pump- and carbapenem resistance-associated outer membrane porin-encoding genes in clinical isolates of *Acinetobacter* species. We analyzed expression of all characterized and as yet uncharacterized RND pump-encoding genes in *Acinetobacter* species, and also analyzed the expression of all three porin-encoding genes known to be involved in the antibiotic resistance of *Acinetobacter* species. Although we did not observe any correlation between the expression of RND pump- and porin-encoding genes, most likely due to the presence of other resistance mechanisms, we conclude that the overexpression of RND pump-encoding genes and downregulation of porin-encoding genes appeared to be quite common in clinical isolates of *Acinetobacter* species from Canadian hospitals.

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