BIOFILM, ICE RECRYSTALLIZATION INHIBITION AND FREEZE-THAW PROTECTION IN AN EPIPHYTE COMMUNITY

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Microbial communities found on the surface of overwintering plants may be exposed to low temperatures as well as multiple freeze-thaw events. To explore the adaptive mechanisms of these epiphytes, with the objective of identifying products for freeze-protection, enrichment libraries were made from frost-exposed leaves. Of 15 identified bacteria from 60 individual clones, approximately half had ice-association activities, with the great majority showing high freeze-thaw resistance. Isolates with ice nucleation activity and ice recrystallization inhibition activity were recovered. Of the latter, two (*Erwinia billingiae* J10, and *Sphingobacterium kitahiroshimense* Y2) showed culture and electron microscopic evidence of motility and/or biofilm production. Mass spectrometric characterization of the *E. billingiae* extracellular polymeric substance (EPS) identified the major proteins as 35 kDa outer membrane protein A and F, supporting its biofilm character. The addition of the EPS preparation increased the freeze-thaw survival of the more susceptible bacteria 1000—10 000 times, and protection was at least partially dependent on the protein component.

Applied microbiologists have long appreciated the biochemical adaptations of microorganisms living under extreme conditions due to their specialized products that have found many applications [1]. However, the acquisition of freeze-thaw survival strategies and the utility of these associated products have received less attention. Depending on the species, bacteria can respond to low temperatures by altering their membrane lipid constitution to maintain fluidity, and the synthesis of cold-shock proteins, chaperones and cold-acclimation proteins, including well-adapted enzymes [2—5]. A few strains produce such highly specialized products as ice nucleation proteins that prevent the supercooling of water, allowing freezing at temperatures close to 0°C [6—7]. Other microbial species inhibit ice recrystallization (IR), which can be associated with the synthesis of antifreeze proteins [8—12] or the production of extracellular polymeric substance (**EPS**) [13—15]. EPS production has been associated with the enhanced survival of marine bacteria at low temperatures [16]. Together, EPS and microorganisms make up biofilms which are well known to confer resistance to a variety of stressful challenges including antibiotics, osmotic changes, nutrition limitations, phagocytes and radiation exposure [17—19].

Although previous studies are not extensive, bacteria exhibiting high freeze-thaw resistance and IR inhibition appear to facilitate the survival of more susceptible species, suggesting that the low temperature adaptations may not only benefit the host bacterium, but also could be useful for members of the consortium [20]. In soil-derived communities, however, it is unknown if these commensurate bacteria are actually associated since sample collections were composited. The aim of the study is to assess the low temperature adaptations and biochemical properties of a community from frost-exposed perennial leaves, and to determine if their products could have utility for the low temperature protection of beneficial species or processes.

MATERIALS AND METHODS

Leaf samples and culture conditions. Leaves of *Chrysanthemum indicum* (cultivar "Arizona Sun") were collected in Kingston Ontario, in mid January, after 81 days of low temperatures (<0°C on a diurnal basis; http://www.weatheroffice.gc.ca/canada_e.html). After washing with sterilized water, the leaves were ground with a mortar and pestle in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and serially-diluted homogenates were cultured in 50 ml of 10% tryptic soy broth (TSB; Difco, USA) or on 10% TSB agar, at 22°C overnight. Liquid cultures were used to construct microbial community libraries (see below) and, as well, individual colonies were examined with those of distinct morphology streaked on 10% TSB agar to obtain single isolates. Control strains, *P. syringae* B728a and *E. coli* TG2, were provided by Dr. D. Guttman and Dr. G. Voordouw, respectively.

Clone library construction and analysis. DNA was isolated from both community liquid cultures and

from single isolates using a phenol-chloroform method [21]. The 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR) from the purified genomic DNA with "universal" bacterial primers (8f: 5'-AGAGTTTGATCCTGGCTCAG and R1406: 5'-ACGGGCGGTGTGTAC; [22]). Amplification was performed at 94°C for 5 min, followed by 35 cycles of 94°C for 0.5 min, 55°C for 0.5 min and 72°C for 2 min, and finally 30 min at 72°C. After electrophoretic separation of the amplified products on 1% agarose gels, the sequences were purified using a nucleotide removal kit (Qiagen, ON, Canada), cloned into pGEMT vector system and subsequently transformed to generate the 16S rRNA gene libraries (Promega, CA, USA). Randomly-selected individual plasmids from the enrichment cultures (48 total) as well as those derived from single colonies (12) were PCR-amplified as above, sequenced and putatively identified by comparison to database sequences using BLAST (National Center for Biotechnology Information web site and the Genbank database; www.ncbi.nlm.nih.gov).

Ice-association and resistance assays. Ice nucleation activity (INA) assays were performed as previously described [23], using *Pseudomonas syringae* B728a as a positive control. Nucleation was assessed by measurement of the heat of crystallization (1 ml samples) and with crossed-polarized images (2 µl). IR inhibition assays were performed as previously described [20] by comparing ice crystal size immediately after freezing (−50°C) and after annealing overnight (−6°C) as indicated [24]. INA and IR inhibition assays were routinely repeated three or more times.

Stationary phase cultures ($\sim 10^8$ cells/ml) were treated with multiple freeze-thaw cycles using a custom cryocycler [20]. Bacterial viability was assessed after 12 and 24 freeze-thaw cycles, with each cycle consisting of 1 h at −18°C and 1 h at 5°C. Numbers of viable bacteria were determined as colony forming units (CFU) on 10% TSB agar incubated at 22°C for 144 h and normalized to an initial count of 1×10^8 cells/ml. For testing the effect of EPS on isolate freeze-thaw viability, the EPS preparations (see below) were UV-sterilized for 30 min prior to addition to cultures (0.8 ml). Some EPS preparations (0.2 ml) were treated with proteinase K (1 mg/ ml) or phospholipase C (40 U/ml) for 60 min at 37°C and subsequently heated (30 min at 65°C) to inactivate the added enzymes. Control experiments included EPS preparations without enzyme addition, medium alone, and *E. coli* TG2 cultures. Freeze-thaw viability was routinely assayed in triplicate, but experiments using purified preparations were done in duplicate.

Adhesion, motility and electron microscopy. Selected isolates and *E. coli* TG2 controls were assessed for their adhesion to polyvinyl chloride (PVC) as previously described [25, 26]. Stationary phase cultures were diluted (1%) with 10% TSB and cultured at 22°C for 48 h or 72 h. After adding crystal violet (0.5% final concentration) and incubating for 15 min, the medium and any unattached bacteria were decanted and the wells were rinsed 5 times with distilled water. Adhering dye was then extracted from the wells in the PVC microtitre plates with 95% ethanol (200 µl) and the absorbance was determined spectrophotometrically at 595 nm.

Motility was assessed on a modified semi-solid *Azotobacter* medium (g/l): $KH_2PO_4 − 0.2$; $K_2HPO_4 − 0.8$; $MgSO_4 − 0.2$; $CaSO_4 − 0.1$; yeast extract − 0.5; mannitol − 2, and a trace of Na_2MnO_4 and $FeCl_3$ in different agar or agarose concentrations, as indicated previously [26]. Swimming, swarming and twitching assays were conducted on 0.3% agarose, 0.5% and 1.5% agar, respectively. All motility assays were initiated by the transfer of colonies grown on 10% TSB agar to the appropriate *Azotobacter* medium with sterilized tooth picks and assessed as previously described [26].

Biofilms were examined by scanning electron microscopy (SEM). Selected isolates and control *E. coli* TG2 cultures were streaked on 10% TSB agar and incubated at 22°C for 48 h, prior to transfer to 4°C for 120 h. They were fixed overnight with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), and washed three times for 5 min each with 0.01 M PBS (pH 7.4). Blocks (2 × 5 mm) were cut, post-fixed for 2 h at 22°C in 1% osmium tetroxide and washed three times (5 min each) with 0.01 M PBS. The samples were then dehydrated with a graded series of ethanol and dried in a critical-point drying apparatus, and subsequently mounted on stubs, coated with a thin layer of gold and then examined under a S-450 scanning electron microscope (Hitachi, Japan) operated at 20 kV. Images were examined and recorded at magnifications of 5000×, 10000× and 20000×.

Bacterial strains were examined under a transmission electron microscope (TEM) after negative staining with phosphotungstic acid (PTA). Briefly, overnight cultures of bacterial cells in 10% TSB ($\sim 1 \times 10^8$ cells/ml) were centrifuged at 10,000 g for 10 min. The pelleted cells were washed three times in 1 ml of 0.01 M PBS, with centrifugation as described above each time and then washed twice in 50 mM $MgSO_4$ (pH 7.4; 50 µl). The washed cells were negatively stained by mixing the cell suspension (10 µl) with 2% PTA (pH 7.0; 2 µl) for 1 min. After transfer of a drop of cell suspension to Formvar-coated nickel grids and absorbing excess liquid with a small piece of Whatman filter paper, the grids were air-dried at room temperature. The cells were viewed on H-7000 electron microscope (Hitachi, Japan) operated at 75 kV and representative photomicrographs captured (15,000× magnification).

EPS preparation and analysis. Single colonies of *E. billingiae* J10 were used to inoculate *Azotobacter* broth medium (10 ml) and after 24 h at 22°C, these cultures were used to inoculate the same medium in shake flasks (4 l). After 120 h at 22°C, the cultures were transferred to 4°C for 1 week, then they were centri-

fuged (8000 *g* for 60 min) and the resulting supernatants added to two volumes of 95% ethyl alcohol or, alternatively, 60% saturated ammonium sulfate. After precipitation overnight at 4°C and centrifugation (8000 *g* for 90 min), the pellets were resuspended in distilled water and dialyzed against 6 changes of distilled water over several days. The dialyzate was then lyophilized and resuspended in distilled water (2 ml). Proteins were separated on 12.5% SDS-PAGE gels and subsequently visualized by staining with Coomassie Blue G250 (Sigma, Canada). Protein concentration was determined using a bicinchoninic acid assay (BCA; Thermo Scientific, USA) with bovine serum albumin as the standard. Polysaccharide concentration was determined with the phenol-sulfuric acid assay using glucose as reference [14]. This preparation was used to investigate its susceptibility to proteinase K and phospholipase C as described in the ice-association and resistance assays above.

Further characterization of the EPS preparation was undertaken by excising and crushing the most prominent band (~35 kDa) from the SDS-PAGE gels. Gel pieces were destained (25 mM ammonium bicarbonate in 50% acetonitrile solution) and treated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate at 56°C for 1 h. After subsequent incubation with 55 mM iodoacetamide at 24°C for 45 min, the gel particles were dried with a SpeedVac centrifuge and subjected to protease digestion in 25 mM ammonium bicarbonate containing 1 ng/μl sequencing grade trypsin (Roche, Canada), for 18 h at 37°C. Peptide fragments were sequentially extracted with 0.1% formic acid, followed by 60% acetonitrile/0.1% formic acid, and then pure (100%) acetonitrile, dried as described above, and desalted using C18 Zip Tips (Millipore, USA).

MALDI QqTOF mass spectrometry. Matrix-assisted laser desorption ionization (MALDI) of the purified EPS protein samples was performed on an Applied Biosystems/MDS Sciex QStar XL Quadrupole Time-of-Flight (QqTOF) mass spectrometer equipped with a oMALDI II source and a nitrogen laser (337 nm). The samples were prepared at a ratio of 1 : 1 (v/v) of the peptide digest to matrix (i.e. 2,5-dihydroxybenzoic acid), and then deposited on a stainless steel MALDI plate. After MALDI MS mapping, peptide sequences were analyzed by tandem mass spectrometry (MS/MS) using argon as the collision gas. The resulting peptide masses were searched against the National Center for Biotechnology Information database using the MS-Fit program with ProteinProspector at the UCSF web site (http://prospector.ucsf.edu), and the sequences were identified by the Mascot search engine (Matrix-Science; http://www.matrixscience.com) based on the MS/MS ions on each spectrum. Search parameters were set to allow two missed trypsin cleavage sites, and the possible modifications of methionine oxidation, carbamidomethylation, deamidation of asparagine and glutamine, and N-terminal pyroglutamation.

Mass tolerance between calculated and observed masses in database search was considered in the range of ±100 ppm for the MS peaks and ±0.2 Da for the MS/MS fragment ions.

RESULTS AND DISCUSSION

An overwintering epiphyte consortium could theoretically allow survival of different species by the sharing of extracellular macromolecules, even though individual bacteria may not be adapted for survival. Fifteen different identified sequences (with 16S rRNA sequence ≥97% identity to the strains in the data bases) were obtained from 60 recovered individual library clones and isolates and approximately half of them had ice-association activities (Table 1). The *P. syringae* J6 isolate had INA comparable to the nucleation activity (type I; mean freezing point ≤2°C) observed in the *P. syringae* B728a controls. IR inhibition, as evidenced by the retention of small size ice crystals after overnight annealing at −6°C, was observed in 6 isolates including *Rahnella* sp.W11, *Duganella zoogloeoides* Y11, *Erwinia billingiae* J10, *Sphingobacterium kitahiroshimense* Y2, *Chryseobacterium* sp. J3, and *Flavobacterium/Chryseobacterium* sp. Yin (Table 1). Revised classification places *Flavobacterium* in the genus *Chryseobacterium* and thus this isolate has been renamed to reflect this. It should be noted that ice-association phenotypes are relatively uncommon. When a portion of the isolates with ice-associating activities were investigated for freeze-thaw resistance, all showed higher levels of freeze-thaw survival than the *E. coli* controls, which lost 7 orders of viability after 12 freeze-thaw cycles, and with no living cells after 24 cycles (Fig. 1; Table 1). In contrast, three of the isolates with IR inhibition activity (*S. kitahiroshimense* Y2, *E. billingiae* J10 and *Flavobacterium/Chryseobacterium* sp. Yin) showed little loss in viability after 36 freeze-thaw cycles (Fig. 1), and less than a ~3 order of magnitude decline (CFU/mL) after 48 cycles (not shown). These observations are consistent with a report of a soil *Chryseobacterium* sp. C14 with IR inhibition that was remarkably freeze-thaw hardy [20]. Notwithstanding, one of the tested epiphytes, *P. syringae* J6, was more susceptible − it lost 7 orders of viability by 24 freeze-thaw cycles and was not viable after 36 cycles (Fig. 1).

We speculated that the *P. syringae* J6 isolate and other frost-susceptible bacteria might depend on affiliation with the epiphyte community for freeze-thaw survival. Three of the identified isolates (*S. kitahiroshimense, E. billingiae*, and *Chryseobacterium* sp.) grew well in culture, with the prospect of practical utility, and therefore these bacteria were selected for further characterization. Resistance to environmental stress can be associated with biofilms [19] and, therefore, these isolates were tested for properties often allied with biofilm production including adherence, motility and matrix affiliation. Motility and biofilm formation

Table 1. Fifteen isolates obtained from 60 sequences derived from enrichment cultures and single colonies from frost-exposed perennials, their closest matches and their ice association activities

Isolate	Closest BLAST Match	Identity, %	Ice-association activity*	Freeze-thaw activity**
Pseudomonas syringae J6	CP000075.1	99	INA	+
Erwinia billingiae J10	Y13249.1	98	IRI	+++
Flavobacterium/Chryseobacterium sp. Yin	AM177621.1	99	IRI	+++
Sphingobacterium kitahiroshimense Y2	AB361248.1	98	IRI	+++
Chryseobacterium sp. J3	DQ530158.1	99	IRI	+++
Rahnella sp. W11	AB476622.1	100	IRI	nt
Duganella zoogloeoide Y11	NR_025833.1	99	IRI	nt
Geobacterium sp. J6	AM712156.1	100	NA	nt
Massilia aurea J9	AM231588.2	99	NA	nt
Sphingomonas sp. J2	AY661593.1	100	NA	nt
Janthinobacterium sp. L1	GQ179711.1	99	NA	nt
Pseudomonas sp. L14	GQ179727.1	99	NA	nt
P. fluorescens L37	AM933520.1	99	NA	nt
P. trivialis L48	FJ179366.1	99	NA	nt
P. veronii L32	AY179328.1	99	NA	nt

* Ice-associating activity is classified as ice nucleating activity (INA), ice recrystallization inhibition (IRI), or not active in these assays (NA).

** Freeze-thaw activity is classified as + for the presence of viable cells after 24 freeze-thaw cycles, ++ for the presence of viable cells after 48 cycles and +++ for cultures showing a decrease in one to three logs of viability after 48 cycles. Not tested cultures are indicated as nt.

are characteristically found together in certain bacteria such as *E. carotovora* where it has been suggested that flagella-derived motility is important for biofilm establishment and morphology [27].

E. coli controls and *Chryseobacterium* sp. were the least adhesive of the isolates with the epiphyte showing a 15% reduction in crystal violet staining ($p < 0.05$ compared to the controls) and with no evidence of motility. In contrast, *E. billingiae* and *S. kitahiroshimense* showed adherence (with means of >44% crystal violet staining over controls; $p < 0.05$). However, only the *E. billingiae* cells could be classified as highly mo-
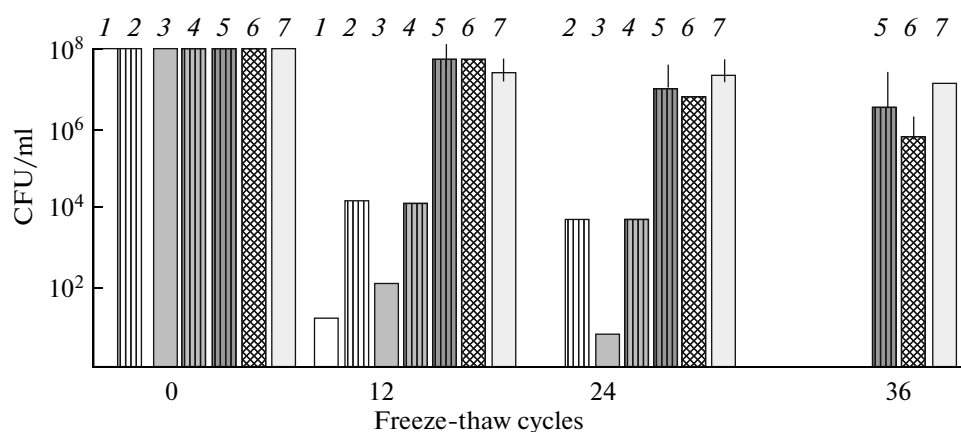

Fig. 1. Freeze-thaw resistance of chosen isolates derived from frost-exposed perennial leaves. Control *E. coli* TG2 (*1*), *P. syringae* J6 (*3*), *E. billingiae* J10 (*5*), *Chryseobacterium* sp. Yin (*6*), and *S. kitahiroshimense* Y2 (*7*), were subjected to 12, 24 or 36 freeze-thaw cycles with viability (CFU/ml) normalized to the concentration at the stationary stage (1×10^8). Standard errors of means are shown only if the error bars are sufficiently large to be depicted. Viability of cultures with the addition of the *E. billingiae* J10 EPS preparation (20%) was shown as *E. coli* TG2 + EPS (*2*), and *P. syringae* J6 + EPS (*4*) for 12 and 24 freeze-thaw cycles, with the bars placed to the right of those representing cultures with no added EPS (*1 vs 2* and *3 vs 4*).
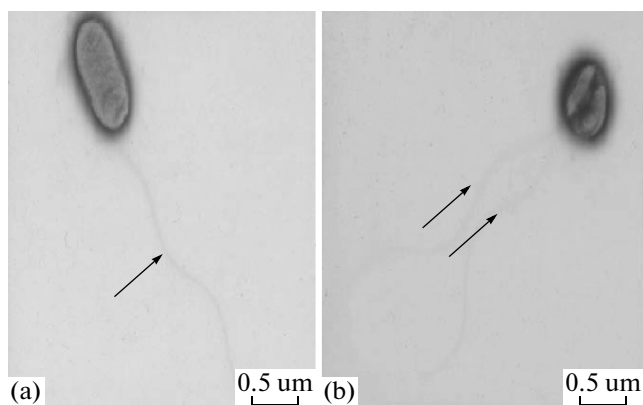
Fig. 2. Representative transmission electron micrographs of the *E. billingiae* J10 cultures stained with phosphotungstic acid. A monotrichous polar flagellum is marked with an arrow (a), and a dividing cell is shown on (b). Other isolates did not show flagella and thus are not shown.

visualized on *E. billingiae,* with two flagella in dividing cells (Fig. 2).

To determine if the adhesive qualities of *E. billingiae* and *S. kitahiroshimense* were associated with biofilm formation, the specimens were examined by SEM. Control *E. coli* cells were similar to published images of this species [28], and there were no obvious extracellular structures associated with *Chryseobacterium* sp. (Fig. 3). *S. kitahiroshimense* showed a limited amount of filamentous material but more than the small amount of matrix seen with *E. coli*, consistent with a low level of biofilm formation. In contrast, SEM examination of *E. billingiae* showed complex extracellular formation that appeared to be composed of matrix material, void space and with 'slimy' structures adhering to the bacteria cells (Fig. 3). Thus, *E. billingiae* showed motility, had a flagellum and macro-colony morphology, and was also affiliated with a complex biofilm-like matrix. Together these adherence and motile phenotypes were similar to those described for other flagellated biofilm producers e.g. [29].

The observed evidence of biofilm formation in *E. billingiae* was further confirmed by biochemical characterization. Fractionation of the *E. billingiae* matrix (using either alcohol or salt precipitation as an initial purification step) resulted in EPS preparations that appeared to be typical of biofilm material, primarily composed of polysaccharides (at a mean concen-
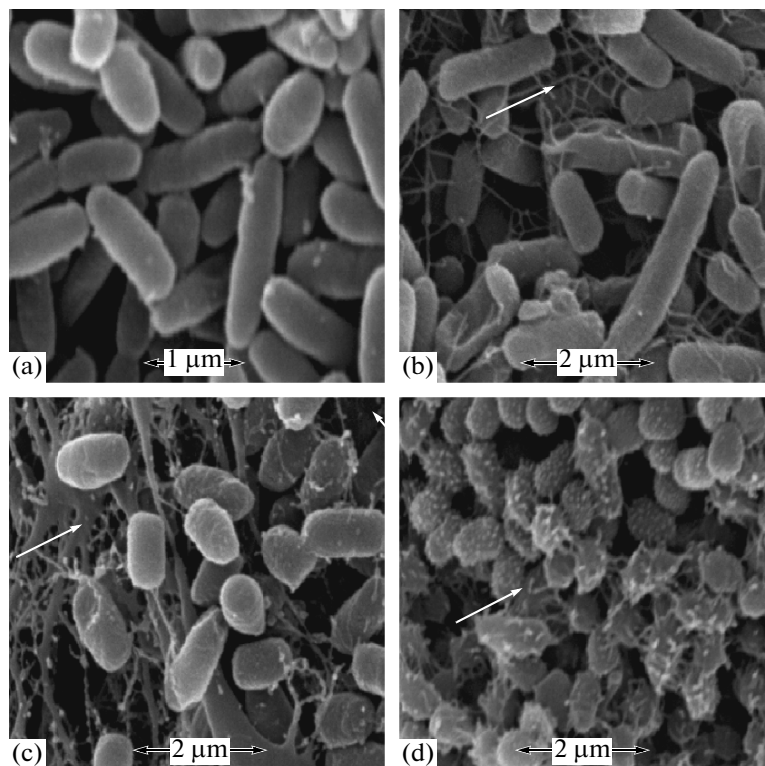
tile on semi-solid medium, with characteristic swimming behavior and distinct swarming rings on appropriate media. Motility assays were verified with TEM observations of negatively stained preparations to confirm the presence or absence of flagella. No flagella were associated with either *Chryseobacterium* sp. or *S. kitahiroshimense* (not shown), but a flagellum was

Fig. 3. Scanning electron micrographs of representative epiphytes including *Chryseobacterium* sp. Yin (a), *E. billingiae* J10 (c) and *S. kitahiroshimense* Y2 (d) as well as control *E. coli* TG2 (b). Arrows indicate representative extracellular matrix structures surrounding *E. billingiae* cells which are less obvious in *S. kitahiroshimense* and *E. coli*.
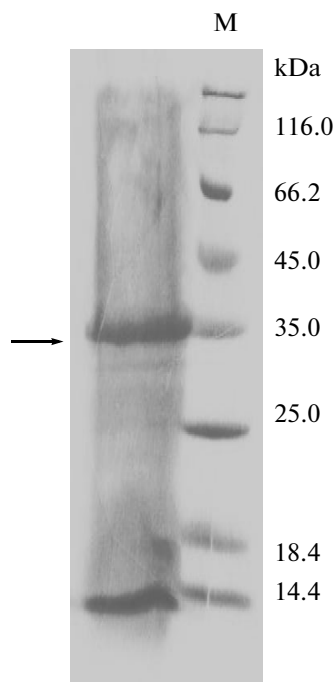
Fig. 4. SDS-PAGE of the partially purified EPS preparation from *E. billingiae* J10 stained for protein with Coomasie Blue. The arrow indicates a major protein band at 35 kDa; a size marker protein mixture was loaded in the lane marked M.

Fig. 5. MALDI QqTOF mass spectrometric analysis of trypsin-digested peptides from the *E. billingiae* J10 EPS preparations. The peptides were derived from the 35 kDa band (as in Fig. 4).

tration of 2.4 mg/ml) and proteins (at a mean concentration of 1.5 mg/ml). Denaturing gel electrophoresis resolved a major 35 kDa protein band and a less-consistent 14 kDa band (Fig. 4). Tryptic digestion and MALDI QqTOF mass spectrometric analysis of the 35 kDa band (Fig. 5) revealed 10 high abundance peptides (Table 2) that showed sequence identity to two *E. billingiae* protein species, corresponding to the outer membrane protein A (OmpA) and outer membrane protein F (OprF), as revealed by the genomic sequence [30]. Both OmpA [31] and OmpF [29] are associated with biofilms. Indeed, strains with disrupted *OmpA* genes have thinner biofilms and are more sensitive to chemical stresses [32].

Having putatively identified the protein components of *Erwinia* biofilms in an isolate that was also characterized by high freeze-thaw survival and IR inhibition (Fig. 1 and Table 1), we investigated the possibility that it could increase the freeze-thaw survival of the other community members. The addition of the *E. billingiae* or *S. kitahiroshimense* cultures to isolates that were relatively freeze-thaw susceptible increased their recovery after 24 freeze-thaw cycles (not shown), but the nature of the conferred protection was difficult to attribute in these complex systems. Therefore, in order to more rigorously investigate any cross-species benefit, the *E. billingiae* EPS preparation was added to two isolates with little evidence of biofilm under the conditions studied, as well as a freeze-thaw susceptible
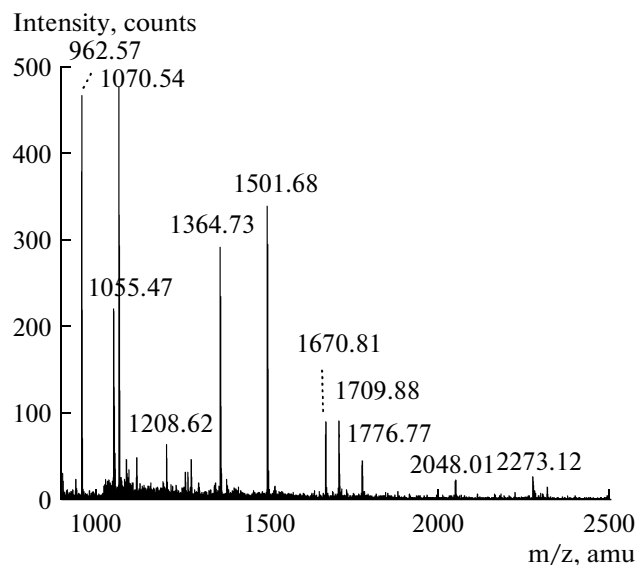
phenotype (Figs. 1 and 3). The viability of *P. syringae* J6 was increased almost three orders of magnitude (from $\sim 10^1$ to $\sim 10^4$ CFU/ml in 24 cycles) with the addition of the *E. billingiae* EPS preparation (Fig. 1). Likewise, the viability of *E. coli* was increased by the addition of the EPS preparation an estimated four orders of magnitude (from no viable cells to 3.1×10^4 CFU/ml). Taken together, the viability of these two cultures increased by 1000–10000 times after 24 freeze-thaw cycles, suggesting that biofilm components could contribute to the overall freeze-thaw resistance of the epiphyte community. At least some of this protection appeared to be associated with protein since limited proteolysis of the EPS preparation resulted in a 10-fold reduction (to 3.1×10^3 CFU/ml) in the recovery of the freeze-thaw susceptible *E. coli* culture after 24 cycles, compared to experiments using the untreated *E. billingiae* EPS. In contrast, phospholipase digestion only reduced the apparent protection about two-fold (to 1.3×10^4 CFU/ml).

Certain epiphytes are associated with biofilm production [33], and as mentioned earlier, biofilms and EPS are often associated with stress resistance [19]. Nevertheless, the relationship between biofilm formation and freeze-thaw resistance is not as well known. It is possible, though, that biofilms act as hydrocolloid-like stabilizers to not only prevent the migration of forming ice crystals but also to reduce water mobility and thereby reduce the probability of IR [34]. Indeed, we speculate that the biofilm of *E. billingiae* could act similarly to the biofilm-associated xanthan gum of *Xanthamonas* sp., which is used by the food industry as a thickener and to inhibit IR [13]. Further support is

Table 2. MALDI QqTOF MS/MS analyses of the tryptic digest of the 35 kDa protein band purified from the extracellular polymeric substance of *E. billingiae* J10, showing correspondence (as indicated by the matches between the measured m/z of the 35 kDa peptides and the calculated masses of the protonated peptides in the data base) to peptides from two genes in the sequenced genome of *E. billingiae*, strain Eb 661 [30]

m/z*	[MH]+**	Delta, Da	Position	Peptide sequence
Outer membrane protein A (gi|299061956, *E. billingiae*, Eb661)				
1055.47	1055.47	−0.00	25—33	DNTWYTGAK
1070.54	1070.55	−0.01	230—238	SDVLFTFNK
1208.62	1208.65	−0.03	289—299	AQSVVDYLVSK
1364.73	1364.75	−0.02	288—299	RAQSVVDYLVSK
1670.81	1670.82	−0.01	109—122	LSYPITDDLDVYTR
Outer membrane protein F (gi|300716100, *E. billingiae*, Eb661)				
962.57	962.58	−0.01	101—109	TRLGFAGLK
1501.68	1501.69	−0.01	322—333	YFEVGATYYFNK
1709.88	1709.91	−0.03	352—368	LGIGSGDTVALGLVYQF
1776.77	1776.81	−0.04	241—256	YDANNVYLAASYAEGR
2048.01	2048.04	−0.03	261—281	ISGNIIDVNGASNAVSGFANK

* The measured mass to charge ratio (m/z).

** The calculated average mass for the protonated species.

provided by previous studies that have shown an association of bacterial EPS and ice [14], with links established between biofilms and resistance to cold and osmotic stress [13, 14, 34].

By themselves, the individual epiphytes in culture showed a range of freeze-thaw resistance (Fig. 1), but as a community, they had survived freeze-thaw stress conditions for months. Since biofilms can accommodate multiple bacteria on a substrate [33] and are associated with environmental stress resistance, it appears reasonable that at least some EPS-producing members could facilitate the persistence of other epiphyte community members. Our study highlights a number of mechanisms involved in the overwintering of an epiphyte community, even for that proportion of the consortium that is culturable, but it also emphasizes that the adaptive properties of a particular species may also serve to support the overall freeze-thaw resistance of microbes in the same niche. One of these, a component of the *Erwinia* EPS, has been partially characterized and the way is now clear to test it for its utility for the low temperature protection of beneficial species or other applied processes. The dramatic increase in the survival of *P. syringae* with EPS (Fig. 1) is of particular note since this species is an efficacious biological control agent with activity against pest fungi, plants and insects (35) but it is not intrinsically very freeze tolerant.

Despite the necessary limitations of the experimentally-dictated survey, this report underscores the wealth of low temperature adaptations including ice-association, freeze-thaw resistance and biofilm formation that all contribute to the complexity found in a single epiphyte community, as well as the potentially useful products that can be identified from such consortia.

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