Production and properties of the native *Chromobacterium violaceum* fucose-binding lectin (CV-III) compared to homologous lectins of *Pseudomonas aeruginosa* (PA-III) and *Ralstonia solanacearum* (RS-III)

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Chromobacterium violaceum is a versatile, violet pigment (violacein)-producing β -proteobacterium, confined to tropical and subtropical regions, dwelling in soil and water, like *Pseudomonas aeruginosa* and *Ralstonia solanacearum*. These three bacteria are saprophytes that occasionally become aggressive opportunistic pathogens virulently attacking animals (the first two) and plants (the third). The recent availability of their genome sequences enabled identification in the *C. violaceum* genome of an ORF (locus no. 1744) that is similar to those of *P. aeruginosa* and *R. solanacearum* lectins, PA-III and RS-III, respectively. A recombinant protein, CV-III, encoded by that ORF exhibited fucose > mannose-specific lectin activity resembling PA-III. This paper describes production and properties of the native CV-III, which, like PA-III and RS-III, is probably also a quorum-sensing-driven secondary metabolite, appearing concomitantly with violacein. Its formation is repressed in the CV026 mutant of *C. violaceum*, which lacks endogenous *N*-acylhomoserine lactone. The upstream extragenic sequence of its ORF contains a 20 bp sequence (5'-101–120) with partial similarities to the *luxI*-box and the related *P. aeruginosa* and *R. solanacearum* promoter boxes of quorum-sensing-controlled genes. The lectin level is augmented by addition of trehalose to the medium. The subunit size of CV-III (around 11·86 kDa) is similar to those of PA-III (11·73 kDa) and RS-III (11·60 kDa). Like PA-III, in the tetrameric form CV-III preferentially agglutinates α 1-2 fucosylated H-positive human erythrocytes (regardless of their A, B or O type), as opposed to the O_h Bombay type, but differs from it in having no interaction with rabbit erythrocytes and in displaying stronger affinity to L-galactose than to L-fucose. The greater similarity of CV-III to PA-III than to RS-III might be related to the selective adaptation of both *C. violaceum* and *P. aeruginosa* to animal tissues versus the preferential homing of *R. solanacearum* to plants.

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INTRODUCTION

Chromobacterium violaceum is a versatile Gram-negative β -proteobacterium (Dewhirst *et al.*, 1989) that produces the violet non-diffusible antibiotic pigment violacein (Lichstein & Van de Sand, 1945; Antonio & Creczynski-Pasa, 2004) and additional antibiotics and enzymes affecting viruses, bacterial and eukaryotic cells (Duran & Menck, 2001; Melo *et al.*, 2003; Chernin *et al.*, 1998). This bacterium is a

common inhabitant of soil and water confined to tropical and subtropical regions. Generally, it behaves as a saprophyte, but sporadically it becomes an aggressive opportunistic animal (including human) pathogen, causing serious infections with a high mortality in immunodeficient individuals (Pemberton *et al.*, 1991; Shao *et al.*, 2002; Alves de Brito *et al.*, 2004). In that behaviour, *C. violaceum* resembles the green pigment (pyocyanin)-producing bacterium *Pseudomonas aeruginosa*, which is a worldwide inhabitant of similar ecosystems and has become an important common nosocomial pathogen, endangering hospitalized chronic patients. Apart from their clinical and pharmacological significance, these two bacteria contribute to environmental bioremediation (Faramarzi *et al.*, 2004).

Abbreviations: AHL, *N*-acylhomoserine lactone; CV-III, *Chromobacterium violaceum* lectin; PA-III, *Pseudomonas aeruginosa* first (galactose-binding) lectin; PA-III, *P. aeruginosa* second (fucose > mannose-binding) lectin; QS, quorum sensing; RS-III, *Ralstonia solanacearum* second (mannose > fucose-binding) lectin.

Since the production of violacein is quorum sensing (QS)-driven, it has become an important tool for bacterial QS signal bioassays, especially for the *N*-acylhomoserine lactone autoinducers (AHLs). A *C. violaceum* mini-Tn5 mutant, CV026 (dependent on exogenous AHL for violacein production), is used as an indicator organism (McClean *et al.*, 1997; Shaw *et al.*, 1997). Such assays are important because AHLs that belong to the class of furanone derivatives (Uroz *et al.*, 2003; Martinelli *et al.*, 2004) are involved in cell-to-cell signalling in Gram-negative bacteria (Fugua *et al.*, 1994; Swift *et al.*, 1999), leading to production of antibiotics, hydrolytic exoenzymes, virulence factors and lectins (Gilboa-Garber *et al.*, 2000; Winzer *et al.*, 2000), all of which contribute to their pathogenicity.

Although the unveiling of the complete genome sequence of *C. violaceum* revealed its remarkable and exploitable adaptability (Brazilian National Genome Project Consortium, 2003), the strategies underlying its adaptability to human infections have not yet been deeply investigated at the molecular and genetic levels (Uroz *et al.*, 2003).

Comparison of the ORFs of *C. violaceum* with those of other organisms revealed closest similarity, 17.4%, to ORFs of the soil-borne phytopathogen *Ralstonia solanacearum* (Salanoubat *et al.*, 2002) and 9.61% similarity to ORFs of the soil-borne animal (including human) pathogen *P. aeruginosa* (Stover *et al.*, 2000). One of the *C. violaceum* homologous ORFs was found to resemble the ORFs of two lectins: *P. aeruginosa* PA-IIL (fucose > mannose; *lecB*) (Gilboa-Garber *et al.*, 2000) and *R. solanacearum* RS-IIL (mannose > fucose) (Sudakevitz *et al.*, 2004).

P. aeruginosa produces PA-IIL (11.73 kDa) (Gilboa-Garber, 1982; Garber *et al.*, 1987) in addition to a galactophilic lectin, PA-IL (12.76 kDa) (Gilboa-Garber, 1972). Both lectins are composed of four identical subunits (Mitchell *et al.*, 2002; Cioffi *et al.*, 2003; Imberty *et al.*, 2004). Their production is controlled together with the bacterium's virulence factors (Gilboa-Garber, 1997) by QS and by RpoS (Winzer *et al.*, 2000), and putative *luxI*-box type elements (transcriptional regulator-binding sites, which are sensitive to the combined stimulatory effects of AHLs and regulator proteins involved in the control of bioluminescence in *Vibrio fischeri*) were identified upstream of their promoter regions. Our suggestion long ago that PA-IL and PA-IIL contribute to the adhesion and biofilm formation of *P. aeruginosa* (Gilboa-Garber, 1997), enabling its pathogenicity (Gilboa-Garber & Garber, 1989), was recently confirmed (Diggle *et al.*, 2002; Tielker *et al.*, 2005).

R. solanacearum possesses a PA-IIL-like lectin (RS-IIL), but does not produce a PA-IL-like one (Gilboa-Garber *et al.*, 2000; Sudakevitz *et al.*, 2004). RS-IIL, which is shorter than PA-IIL by one amino acid (11.60 kDa), resembles it in its composition, Ca²⁺ requirement for activity and a very high sugar affinity (Garber *et al.*, 1987; Sudakevitz *et al.*, 2004). However, it differs from PA-IIL in showing highest affinity

to mannose, accompanied by a lower avidity for fucose and related sugars (Sudakevitz *et al.*, 2004).

The ORF of the PA-IIL- and RS-IIL-like putative *C. violaceum* lectin (named CV-IIL) was used for production of a recombinant protein that was shown to display a preferential fucose-binding activity (Wimmerova *et al.*, 2005; M. Wimmerova & A. Imberty, unpublished) and a native fucose-binding lectin was found in *C. violaceum* cell extracts (K. Zinger-Yosovich & N. Gilboa-Garber, unpublished). No PA-IL-like ORF was found in the *C. violaceum* genome, nor PA-IL-like activity in its cell extracts.

In the present study, native CV-IIL production was examined using both wild-type and the CV026 mutant. The properties of the purified native CV-IIL, isolated from the wild-type, and its interactions with human and animal erythrocytes and with various monosaccharides and EDTA were compared to those of PA-IIL and RS-IIL.

METHODS

Bacterial strains and cultures. *C. violaceum* wild-type ATCC 12472, and *R. solanacearum* ATCC 11696 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *P. aeruginosa* ATCC 33347 is our isolate deposited at ATCC and *C. violaceum* CV026 violacein-negative mini-Tn5 mutant of ATCC 31532 was kindly provided by Professor P. Williams, University of Nottingham, UK.

All the bacteria were grown at 28 °C with vigorous shaking for 3 days in nutrient broth and supplied with 0.4% trehalose. The harvested cells were washed three times and disintegrated by sonication.

Purified lectin preparations. The preparation of the bacterial (wild-type) cell extracts and the procedures for purification of the three lectins, CV-IIL, PA-IIL and RS-IIL, including heating to 65 °C for 15 min, ammonium sulfate precipitation (70% saturation), and affinity chromatography using Sepharose-mannose, were performed as earlier described (Gilboa-Garber, 1982; Sudakevitz *et al.*, 2004). The lectins were eluted from the column by 0.1 M EDTA, followed by an overnight dialysis against saline containing 0.01 M CaCl₂, followed by another similar dialysis against either PBS (for the haemagglutination tests) or twice-distilled water (for other purposes and for concentration of the preparations). The purified lectin preparations were analysed by SDS-PAGE with Coomassie brilliant blue staining, as previously described (Sudakevitz *et al.*, 2004).

N-terminal amino acid sequencing and mass spectrometry analysis. The purified *C. violaceum* lectin preparation, giving a single band in SDS-PAGE, was subjected to N-terminal amino acid sequence determination by Edman degradation and to mass spectrometry by the Technion Protein Research Center, as previously described (Sudakevitz *et al.*, 2004).

Analyses of the upstream extragenic sequences of the CV-IIL ORF. Homology searching of the upstream (200 bp fragment) sequences of the genes (beginning from their transcription starts) of CV-IIL (locus name CV 1744, <http://www.brgene.lncc.br/cviolaceum>) and RS-IIL (locus name RSc 3288), was performed as described before for PA-IL and PA-IIL (Gilboa-Garber *et al.*, 2000).

Haemagglutination and tests for its inhibition. Diverse human and animal erythrocytes (kindly supplied by the Magen David Adom National Blood Services in Israel and the animal centre of

Bar-Ilan University, Israel) were washed three times with phosphate-buffered (0.025 M, pH 7.2) isotonic NaCl solution (PBS) and then treated by 0.1% papain with 0.01% cysteine, as previously described (Gilboa-Garber, 1982; Sudakevitz *et al.*, 2004). A 50 μ l sample of each lectin preparation examined was serially diluted with 50 μ l PBS to produce twofold dilutions. PBS and erythrocyte suspensions (50 μ l each) were added to each tube. After 30 min at room temperature, the tubes were centrifuged for 30 s (1000 g), and the haemagglutinating activity was examined as previously described (Gilboa-Garber, 1982). The activity was represented by the number of tubes in which there was haemagglutination, along the twofold dilutions (e.g. 7 positive tubes = positive reaction up to dilution of 1:128, or original activity of 128 haemagglutination units).

In the haemagglutination inhibition test, each solution examined (carbohydrates at 0.3 M or EDTA at 0.1 M) was serially twofold diluted in 50 μ l PBS and then 50 μ l of the lectin solution (using the highest dilution leading to agglutination of all the erythrocytes in one large mass) was added to each tube. After 30 min at room temperature, 50 μ l of 5% papain-treated human O blood type erythrocyte suspension was added to each tube (Gilboa-Garber, 1982) and, after another 30 min, the haemagglutination was examined as described above.

The haemagglutination inhibition intensity was represented by the highest number of dilutions ($\log_2$ dilution⁻¹) at which no significant haemagglutination occurred.

Statistical evaluation. The results of the haemagglutination and haemagglutination inhibition tests were analysed by Student's *t*-test.

RESULTS

Haemagglutinating activities of intact *C. violaceum* cells and their extracts and purification of CV-IIL from the extracts

Examination of intact *C. violaceum* cell suspensions revealed considerable haemagglutinating activities of the cell-surface adhesins. Those included strong sugar- and EDTA-insensitive and -sensitive activities. The crude extracts of the CV026 mutant exhibited much lower haemagglutinating activities as compared to those of the wild-type. Since the fucose/mannose- and EDTA-sensitive activity, resembling that of PA-IIL, was the goal of our research, we purified it from the wild-type *C. violaceum* crude cell extracts, as we

did for PA-IIL and RS-IIL, using the procedures described in Methods. In all steps of the purification procedure, the CV-IIL activity behaved like those of PA-IIL and RS-IIL.

Examination of the purified lectin by SDS-PAGE stained with Coomassie brilliant blue (Fig. 1a) revealed the desired single band of CV-IIL with a molecular mass around 11.9 kDa, close to that of PA-IIL.

N-terminal amino acid sequence of the purified native CV-IIL

Analysis of the N-terminal amino acid sequence of the purified lectin revealed two molecule populations. Both of them showed exactly the anticipated sequence, AQQG-VFTLPARINFGVTVLV, but while the majority of molecules did not bear the initiating methionine (113 amino acids), a minority did contain that methionine (114 amino acids).

Mass spectrometric analysis

The above two (major and minor) subunit populations were also observed in the mass spectrometric analysis (Fig. 1b). The molecular mass values registered were: 11 852.6104 Da (without initiating methionine) and 11 978.4834 (with that methionine) Da. This and the above results confirmed that the isolated protein was indeed CV-IIL.

Search for a *luxI*-box-like sequence in CV-IIL and RS-IIL gene promoters

Since CV-IIL and RS-IIL, like PA-IIL, were found to be produced in the late stationary phase of the bacteria (3 days), we looked for *luxI*-box-like sequences upstream of their genes using the *luxI*-box and *luxI*-box-type sequence of PA-IIL (located 24–43 bp upstream of its ORF). That alignment revealed partial sequence similarity 101–120 and 115–134 bp upstream of the transcriptional starts of the CV-IIL and RS-IIL genes, respectively (Fig. 2).

Fig. 1. (a) SDS-PAGE of purified CV-IIL (3) and PA-IIL (2), stained by Coomassie brilliant blue; (b) mass spectroscopic analysis of CV-IIL.

CV-IIL gene	GCCTGACTGG	GCCTGACTGG	7/9
luxI-box	ACCTGTACGA	TGTAACAGGT	8/9
PA-IIL gene	GGCTGCCTGA	AAAGGCAGGC	8/9
RS-IIL gene	ACCTGTACGA	CAGCCGCGCG	9/9
Consensus	CCTG	GA	GC G

Fig. 2. *luxI*-box-like 20 bp nucleotide sequences (possible AHL-binding sites) upstream of the genes encoding CV-IIL (locus name CV 1744), PA-IIL (Gilboa-Garber *et al.*, 2000) and RS-IIL (locus name RSc 3288). The points represent complementary symmetry between the first and second 10 bp segments composing the 20 bp of each box. Identity with *luxI*-box basepairs is indicated by black, and similarity between two or three of the four by grey. The consensus 9 bp include those representing 4/4 boxes (underlined) and those representing 3/4 boxes. The numbers on the right represent the basepairs fitting the consensus 9 bp.

Comparison of the haemagglutinating activities of CV-IIL, PA-IIL and RS-IIL towards human erythrocytes of different ABO types

Using adult human H-positive red blood cells of types O, A, B and AB, it was found that the three lectins agglutinated all of them regardless of their ABO types (Fig. 3). However, when O_h Bombay-type erythrocytes, lacking the α 1-2 fucosyl residue, were examined, CV-IIL, like PA-IIL, agglutinated them very weakly; RS-IIL did interact with those H-negative erythrocytes, although at somewhat reduced intensity. Fetal erythrocytes obtained from cord bloods were agglutinated very well by all three lectins.

Comparison of the haemagglutinating activities of CV-IIL, PA-IIL and RS-IIL towards different animal erythrocytes

Using human (O-type), cow, sheep, rabbit and rat erythrocytes, it became apparent that there are some differences not only between CV-IIL (Fuc > Man) and RS-IIL (Man > Fuc), but also between CV-IIL and PA-IIL, which is closer to it in preferential fucose affinity. As seen in Fig. 4, the three lectins did not react at all with cow erythrocytes while reacting well with human, sheep and rat erythrocytes. However, whereas PA-IIL strongly agglutinated the rabbit, rat and sheep erythrocytes, and RS-IIL most strongly agglutinated the sheep cells followed by the rabbit red blood cells, CV-IIL most strongly agglutinated the rat erythrocytes and did not agglutinate the rabbit cells at all.

Comparison of the monosaccharide specificity and sensitivity to EDTA of CV-IIL and PA-IIL

Comparison of the inhibition the haemagglutinating activities of purified CV-IIL and PA-IIL by EDTA and a series of monosaccharides (Fig. 5) revealed that these two lectins, which do not interact with D-glucose or D-galactose, are very

Fig. 3. Comparison of the haemagglutinating activities of CV-IIL, PA-IIL and RS-IIL towards papain-treated human erythrocytes of different ABO types from adults and from fetal (cord) bloods. The data represent means of at least six experiments $\pm$ SEM.

similar in their EDTA sensitivity and Fuc > Ara > Fru > Man specificity order (with subtle affinity differences). An exception to the order similarity is the higher affinity of CV-IIL to L-galactose than to L-fucose, not observed with PA-IIL or RS-IIL.

DISCUSSION

In the last decade the availability of the whole-genome sequences of the closely related opportunistic pathogenic bacteria *P. aeruginosa* (Stover *et al.*, 2000), *R. solanacearum* (Salanoubat *et al.*, 2002) and *C. violaceum* (Brazilian National Genome Project Consortium, 2003) has enabled comparative studies of the molecular basis, properties and function of their virulence factors and regulation of their production. The central pillars of the virulence arsenals of these bacteria are lectins, which, by virtue of their sugar-binding, contribute to the pathogens' host specificity and adherence to host cells, facilitating the establishment of infection. The first lectins of these bacteria, PA-IIL and PA-IIL, were discovered in *P. aeruginosa* cell extracts (Gilboa-Garber, 1972; Gilboa-Garber 1982) before the genome sequence of this bacterium had been determined. Their gene sequences and regulation of their production have since been studied (Gilboa-Garber, 1997; Gilboa-Garber *et al.*, 2000; Winzer *et al.*, 2000), as well as their 3D structures and their interactions with saccharides (Mitchell *et al.*, 2002;

Fig. 4. Comparison of the haemagglutinating activities of CV-IIL, PA-IIL and RS-IIL towards papain-treated human (O-type) and diverse animal erythrocytes. The data represent means of at least six experiments $\pm$ SEM.

Ciocci *et al.*, 2003; Imberty *et al.*, 2004) for insight into the molecular basis of their host glycan recognition. The existence of RS-IIL, which is homologous to PA-IIL, was deduced following an alignment study comparing the PA-IIL sequence with the newly published *R. solanacearum* genome sequence (Sudakevitz *et al.*, 2004). Recombinant RS-IIL was produced soon after, and both the native and recombinant lectins were analysed. The crystal 3D structure of the lectin interacting with the saccharides for which it has high affinity showed that minute differences in amino acid composition between RS-IIL and PA-IIL determine the variation in their sugar affinities – the preferential affinity of PA-IIL for L-fucose versus that of RS-IIL for D-mannose (Sudakevitz *et al.*, 2004).

With the release of the *C. violaceum* genome sequence, a third homologous lectin was deduced based on alignment search using PA-IIL against the *C. violaceum* genome. The alignment information indicated a homologous ORF of the same size as PA-IIL and RS-IIL, differing from them only in a few amino acids (Sudakevitz *et al.*, 2004). The putative lectin ORF was cloned in *E. coli* and the recombinant CV-IIL protein was found to be a tetrameric lectin which

Fig. 5. Comparison of the inhibition of CV-IIL and PA-IIL by decreasing concentrations of various monosaccharides (beginning at 0.3 M) and EDTA (beginning at 0.1 M). The data represent means of at least six experiments $\pm$ SEM.

resembles PA-IIL in L-fucose preference; its crystal 3D structures in interactions with fucose and mannose derivatives are currently being investigated (M. Wimmerova & A. Imberty, unpublished).

The present study showed that the purified native *C. violaceum* lectin is similar to PA-IIL in its molecular mass (around 11.9 kDa). Two molecule populations (with and without the initiating methionine) are clearly observed. The N-terminal amino acid analysis data fit the predicted sequence and confirm the native CV-IIL homology to PA-IIL and RS-IIL and to the recombinant lectin of Wimmerova *et al.* (2005).

As for the regulation of CV-IIL and RS-IIL formation: (a) these two lectins, like native PA-IIL, are mainly produced (preferentially under osmotically balanced conditions) in the late stationary phase; (b) the *C. violaceum* mutant, CV026, which displays repressed autoinducer formation, produces very low lectin activity under the same growth conditions; and (c) like PA-IIL and PA-IIL, which are known to be under QS cascade control (Gilboa-Garber *et al.*, 2000; Winzer *et al.*, 2000) their gene upstream regions contain *luxI*-box-like 20 bp sequences (Fig. 2) supposed to bind QS signals for their gene expression. Taken together these data may be regarded as an indirect indication that the production of both CV-IIL and RS-IIL, like those of PA-IIL and PA-IIL, might be driven by QS; however, this proposal has to be proven experimentally.

The results of the haemagglutination tests (Figs 3 and 4) show that while CV-IIL resembles PA-IIL (more than

RS-IIL) in selective agglutination of H-positive erythrocytes, it differs from PA-IIL (and RS-IIL) in showing no interaction with rabbit cells.

In accordance with results obtained with the recombinant CV-IIL (Wimmerová *et al.*, 2005; M. Wimmerová & A. Imberty, personal communication), the native lectin exhibits EDTA sensitivity and a very high L-fucose affinity, followed by somewhat lower arabinose, fructose and D-mannose avidities, and does not react at all with either D-glucose or D-galactose (Fig. 5). In its higher fucose versus mannose affinity, CV-IIL is similar to PA-IIL but not to RS-IIL. However, it differs from PA-IIL, and also from RS-IIL, in L-galactose > L-fucose affinity, which is probably related to small amino acid variations that are associated with stronger attraction of the CV-IIL binding site by the 6th CH₂OH of L-galactose while the other two lectins are more strongly attracted by the terminal CH₃ of L-fucose. The amino acid motif 22–24, which is involved in monosaccharide specificity, is Ser-Ser-Gly in PA-IIL, Ala-Ala-Asn in RS-IIL and Ser-Ala-Ala in CV-IIL (Imberty *et al.*, 2004; Sudakevitz *et al.*, 2004).

Conclusions

Native CV-IIL (11·9 kDa) is homologous to PA-IIL (11·7) and RS-IIL (11·6) in its structure and properties, including sugar and EDTA sensitivity, and in the regulation of its production. It is also similar to those lectins in its interactions with H-positive human erythrocytes regardless of their ABO type, and with rat and sheep erythrocytes, but not cow erythrocytes. In its selectivity to H-positive cells and stronger fucose versus mannose affinity, CV-IIL is closer to PA-IIL than to RS-IIL. This similarity might be related to their common host spectrum, which is mainly animal while that of RS-IIL is mainly plant. However, it differs from both PA-IIL and RS-IIL in showing no interaction with rabbit erythrocytes and in its L-galactose > L-fucose preference.

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