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Propionibacterium jensenii Produces the Polyene Pigment Granadaene and Has Hemolytic Properties Similar to Those of *Streptococcus agalactiae*▽†

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The red polyene pigment granadaene was purified and identified from *Propionibacterium jensenii*. Granadaene has previously been identified only in *Streptococcus agalactiae*, where the pigment correlates with the hemolytic activity of the bacterium. A connection between hemolytic activity and the production of the red pigment has also been observed in *P. jensenii*, as nonpigmented strains are nonhemolytic. The pigment and hemolytic activity from *S. agalactiae* can be extracted from the bacterium with a starch extraction solution, and this solution also extracts the pigment and hemolytic activity from *P. jensenii*. A partial purification of the hemolytic activity was achieved, but the requirement for starch to preserve its activity made the purification unsuccessful. Partially purified hemolytic fractions were pigmented, and the color intensity of the fractions coincided with the hemolytic titer. The pigment was produced in a soluble form when associated with starch, and the UV-visual spectrum of the extract gave absorption peaks of 463 nm, 492 nm, and 524 nm. The pigment could also be extracted from the cells by a low-salt buffer, but it was then aggregated. The purification of the pigment from *P. jensenii* was performed, and mass spectrometry and nuclear magnetic resonance analysis revealed that *P. jensenii* indeed produces granadaene as seen in *S. agalactiae*.

Propionibacterium jensenii is a gram-positive bacterium with a high G+C content and is grouped together with other dairy bacteria, *Propionibacterium freudenreichii*, *Propionibacterium thoenii*, and *Propionibacterium acidipropionici*, in the classical division of the propionic acid bacteria (6). The other group of propionic acid bacteria is the cutaneous group, whose members are a part of the bacterial flora of the human skin; these organisms are considered to be opportunistic and have been found to be involved in infections (4, 24). The classical or dairy propionibacteria were originally identified and isolated from raw milk and other dairy products (23). The species primarily found in cheese are *P. freudenreichii* and *P. jensenii* (3, 17, 27), and strains of *P. freudenreichii* are used for the production of Swiss-type cheeses.

The presence of propionibacteria in cheese can cause defects such as splitting (10) and red spots (1). The defect known as "red-spotting" is considered to be a quality problem and is caused by pigmented strains of *P. jensenii* and *P. thoenii*. The defect is for the most part a problem in cheese made from unpasteurized milk. In addition to producing a red pigment, the strains are beta-hemolytic, but they have never been considered to be pathogenic. Vedamuthu et al. (32) observed that only the pigmented strains of *P. jensenii* and *P. thoenii* were hemolytic. Experimental evidence of a link between pigmentation and hemolytic activity was supported by the chemical mutation of *P. jensenii* and *P. thoenii* with 1-methyl-3-nitro-1-nitrosoguanidine (T. Langsrud, unpublished data). Nonpigmented mutants of *P. jensenii* and *P. thoenii* were found to be nonhemolytic, while mutants that showed reduced pigmentation also displayed reduced hemolytic activity.

A link between pigment and hemolytic activity has also been described for *Streptococcus agalactiae* (30). The level of beta-hemolytic activity correlates with the amount of the red pigment produced by the organism (7, 26, 29). Recently, the structure of the pigment was determined (26). It was shown to be an ornithine rhamno-polyene with a linear chain of 12 conjugated double bonds and was assigned the trivial name granadaene. The hemolysin from *S. agalactiae* has never been isolated due to its instability (20).

The aim of this study was to characterize the hemolytic activity and pigmentation of *P. jensenii* and investigate any link between these two properties. In this paper, we report that the pigment from *P. jensenii* is identical to granadaene from *S. agalactiae* and that the two bacteria also display similar hemolytic activities.

MATERIALS AND METHODS

Bacterial strains and media. Strains of *P. jensenii* and *P. thoenii* from our strain collection were tested for hemolytic activity on blood agar. The strains were grown anaerobically at 30°C for 3 to 4 days on agar made from tryptone blood agar base (Oxoid, Basingstoke, England) supplemented with 5% defibrinated horse blood. The strain used for the rest of the study was *Propionibacterium jensenii* LMGT 2818. The bacterium was cultivated anaerobically in sodium lactate broth (SLB) (10% tryptone, 10% yeast extract, 0.8% sodium lactate, and 0.25 g of K_2HPO_4/liter) (17) at 30°C. In some experiments, the SLB was modified by the addition of either 0.2% starch, 1% starch (potato starch; Sigma), or 3% Tween 80.

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Preparation of extracts with hemolytic activity and pigment. Two different methods were used to extract hemolytic activity and pigment from *P. jensenii* LMGT 2818. In the first method, the bacterium was propagated in SLB at 30°C for 48 to 72 h, and the cells were collected by centrifugation at 2,400 × *g* for 15 min at 4°C. Cell pellets were washed twice in phosphate-buffered saline (PBS) and resuspended in an extractor solution which consisted of 1% starch and 3% Tween 80 in PBS (pH 7.4) (19). The suspension was incubated at 37°C for 1 h and subjected to centrifugation at 25,800 × *g* for 30 min, and the supernatant which contained the hemolytic activity and pigment was collected and stored on ice. In the second method, the cells were grown for 48 h at 30°C in SLB modified by the addition of either 0.2% starch, 1% starch, or 20% of the extractor solution. After centrifugation of the culture at 9,600 × *g* for 15 min at 4°C, the supernatant, which contained the hemolytic activity and pigment, was precipitated by the addition of an equal volume of cold methanol (18) and placed on ice for 5 min. The mixture was subjected to centrifugation at 11,000 × *g* at 4°C for 10 min, and the pellet was suspended in 5 mM Tris-HCl (pH 8.0), precipitated again with 1 part cold methanol, and finally resuspended in 5 mM Tris-HCl (pH 8.0) in a volume corresponding to 5% of the initial culture volume. Absorption spectra were measured with a Shimadzu model UV-160 UV-visual (VIS) scanning spectrometer.

Assay for hemolytic activity. Hemolysis was measured as the decrease in the turbidity (optical density at 620 nm [OD_{620}]) of horse erythrocytes (TCS Biosciences Ltd., England) as determined using a microplate reader (Multiskan Ascent; Labsystems). The erythrocytes were washed with PBS two to three times prior to use and diluted to 1% in PBS. One hundred microliters of the erythrocyte solution was applied to the wells of a 96-well microtiter plate. Samples containing hemolytic activity were added to assay wells in volumes of 10 μl, and twofold serial dilutions were made across the wells. For routine analysis, hemolytic activity was assayed at 37°C for 30 min. One hemolysin unit (HU) was defined as the amount of hemolysin eliciting a 50% decrease in the OD_{620} of the erythrocytes.

Samples of bacterial cells were prepared by washing the cells three times in PBS before resuspending the cells in PBS to the original volume, and 10 μl was analyzed for hemolytic activity.

Effects of proteinase K, alpha-amylase, and heat on hemolytic activity. Alpha-amylase (type *Bacillus subtilis*, no. 10069; Sigma) and proteinase K (Promega) were tested for their ability to affect the hemolytic activity. Stock solutions of 20 mg/ml were prepared. Alpha-amylase was dissolved in water and proteinase K in a buffer (50 mM Tris-HCl [pH 8.0], 10 mM $CaCl_2$). Alpha-amylase was added to a final concentration of 0.95 mg/ml to 100 μl of hemolytic extract containing 2,202 HU and incubated at 25°C for 90 min. Proteinase K was added to a final concentration of 0.25 mg/ml to 100 μl of hemolytic extract containing 314 HU and incubated at 37°C for 1 h. Controls consisted of hemolytic extract incubated under the same conditions but without enzyme. For the heat inactivation experiment, a hemolytic extract containing 2,509 HU was incubated at 60°C, and a sample was removed after 10, 20, and 30 min. After the treatments, samples were assayed for hemolytic activity as described above.

Osmotic protection experiments. A 1% horse erythrocyte solution containing an osmotic protectant was mixed with hemolytic extract (36 HU). Polyethylene glycols (PEGs) of different molecular weights were used as osmotic protectants. PEG 1500 (Merck) and PEG 3000 (Merck) were used at a final concentration of 30 mM. PEG 6000 (Merck) and PEG 8000 (Sigma) were used at a final concentration of 15 mM. Hemolysis was assayed by cell turbidity as described above.

Partial purification of hemolysin. A concentrated hemolytic extract prepared according to the second method was applied to a 20-ml HiPrep 16/10 Q XL column (Amersham Biosciences) equilibrated with 10 mM sodium phosphate buffer (pH 7.0). Bound material was eluted with 0.3 M NaCl followed by 1 M NaOH in 3-ml fractions. Active fractions were subjected to phenol extraction to remove starch (http://nu-distance.unl.edu/homer/class/4/Mastery/text/proteinphenol.html). Protein samples were then analyzed by sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis by the method of Laemmli (13) and stained with Coomassie blue.

Extraction and purification of pigment. *Propionibacterium jensenii* was cultured in SLB at 30°C until the culture obtained an OD_{600} of approximately 4.0. The cells were harvested by centrifugation at 9,600 × *g* at 4°C for 15 min. Bacterial cell pellets were then washed with 20 mM sodium phosphate buffer (pH 7.0) and concentrated by centrifugation at 9,600 × *g* for 10 to 15 min. The supernatant, containing the pigment in an aggregated form, was collected, and the washing procedure was repeated two to three times until most of the pigment was removed from the bacteria. The aggregated pigment suspended in the washing buffer was concentrated by centrifugation at 25,800 × *g* at 4°C for 20 min. The pelleted pigment was collected and transferred to Eppendorf microcentrifuge tubes. The pigment was washed twice with distilled water and collected by centrifugation at 16,100 × *g*. The pigment was then washed twice in dimethyl sulfoxide (DMSO) and dissolved in DMSO-0.1% trifluoroacetic acid (TFA). The dissolved pigment was subjected to centrifugation, and the debris pellet was removed. The pigment was then precipitated by the addition of 25% ammonia (analytical grade) to a final concentration of 0.25% in DMSO-0.1% TFA. The pellet was dissolved in DMSO-0.1% TFA and applied to a column (150 by 15 mm) of Sephadex LH-20 (Pharmacia, Uppsala, Sweden) equilibrated in the same solvent. The column was eluted with DMSO-0.1% TFA, and pigmented fractions were collected, precipitated with ammonia as described above, washed three times with distilled water, and dried.

NMR. ^{1}H, one-dimensional, rotating-frame Overhauser enhancement spectroscopy; one-dimensional total-correlation spectroscopy; ^{1}H-^{1}H correlation spectroscopy; ^{1}H-^{13}C heteronuclear single-quantum correlation; and ^{1}H-^{13}C heteronuclear multiple-bond correlation (HMBC) nuclear magnetic resonance (NMR) experiments were performed at 298 K on a Bruker Avance 600 Fourier transform-NMR spectrometer equipped with a TCI CryoProbe. The dried sample was dissolved in dimethylsulfoxide-d_6 (hexadeuterodimethyl sulfoxide) containing 0.2% trifluoroacetic acid-*d*. Chemical shifts were calibrated against residual DMSO-d_5 at 2.50 ppm for ^{1}H and 40.4 ppm for ^{13}C.

MS. Purified dried pigment was dissolved in DMSO-0.1% TFA and analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (TOF MS) analysis. A dried droplet preparation of the sample was applied to a Bruker ground-steel matrix-assisted laser desorption ionization target, undiluted sample was mixed 1:1 with matrix solution (1 part 10-mg/ml 2,5-dihydroxy benzoic acid with 2-hydroxy-5-methoxy benzoic acid in acetonitrile to 2 parts 0.1% TFA), and ca. 0.5 μl was applied to the target and left to dry at ambient temperature. Mass spectra (positive ions) were recorded on a Bruker Daltonics Ultraflex TOF-TOF instrument operated in reflectron mode, with the acceleration voltage set to 25 kV and the delayed extraction to 40 ns. Laser power and detector gain were adjusted to optimize the signal-to-noise ratio and resolution.

RESULTS AND DISCUSSION

Production of hemolytic activity and pigment during growth. Twenty pigmented strains of *P. jensenii* and *P. thoenii* were assayed for hemolytic activity on blood agar. All the strains produced similar zones of beta-hemolysis. The zones appeared after 1 day of incubation at 30°C and continued to increase during the next 3 to 4 days of incubation. Some of the strains of *P. jensenii* and *P. thoenii* had a slimy texture and were difficult to concentrate by centrifugation. The strain *P. jensenii* LMGT 2818 was selected for further studies because of the easy handling of this strain.

Hemolytic activity could not be detected in the culture supernatants from *P. jensenii* propagated in SLB. However, hemolytic activity was detectable in assays with whole bacterial cells grown in SLB. The activity was detectable during all phases of growth and reached a maximum at the onset of stationary phase and then declined (Fig. 1A). To further characterize the hemolytic activity, different extraction protocols were applied to obtain cell-free hemolysin. Some hemolysins require stabilizers to preserve activity, like the hemolysins of *S. agalactiae* (19) and streptolysin S (16). A solution consisting of soluble starch and Tween 80 in PBS, originally designed for extraction of hemolytic activity from *S. agalactiae* (19), was successfully applied to obtain the hemolytic activity from cells of *P. jensenii*. The key ingredient used for extraction was starch. The starch acted as a stabilizer and formed a strong complex with the hemolysin. The hemolytic activity is surface associated, and there seems to be a preference for starch over the natural carrier on the cells. An extraction solution of PBS containing 3% Tween 80 was also effectively used to extract the hemolytic activity, but this extract was unstable and the activity was lost during the day and when stored at 4°C or −20°C.

Propionibacterium jensenii was cultivated under different growth conditions with starch and Tween 80 added to SLB, and

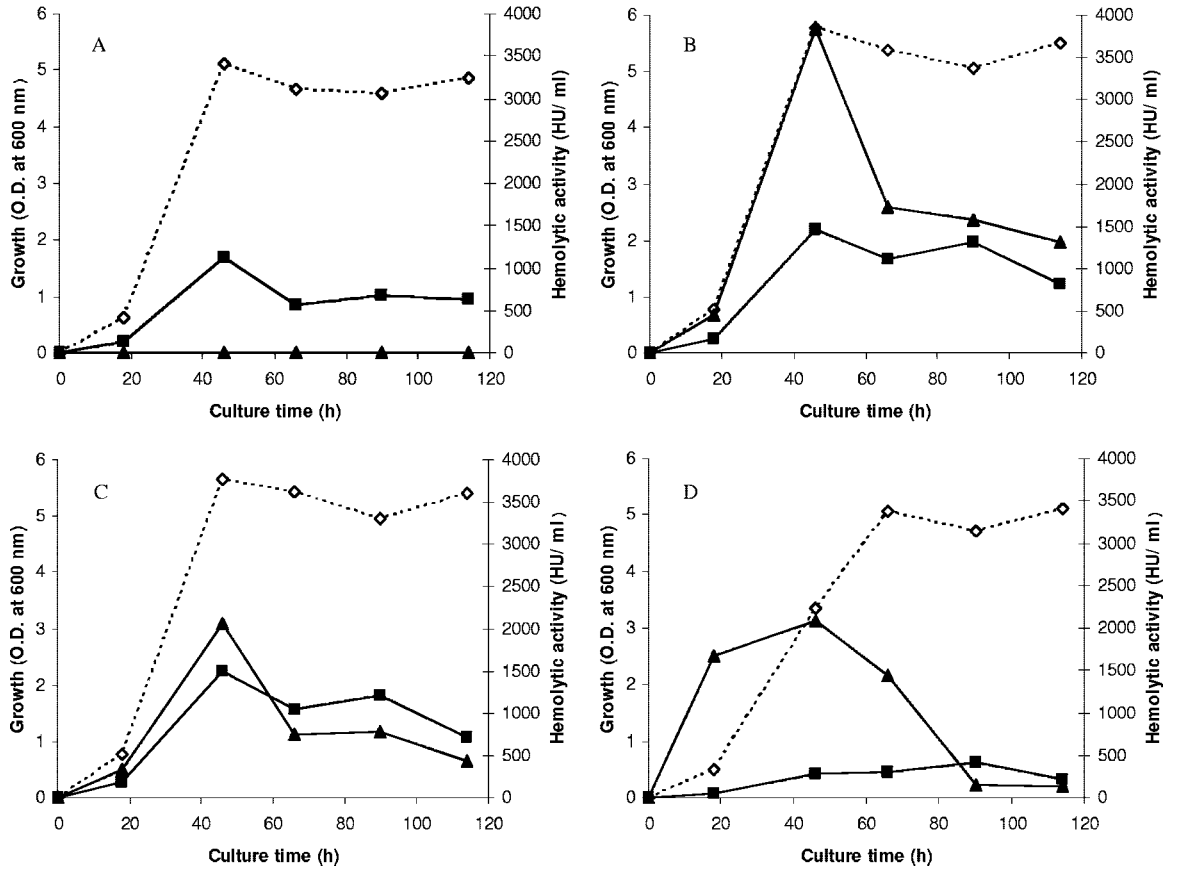

FIG. 1. Growth and hemolytic activity of *P. jensenii* LMGT 2818. The culture media SLB (A), SLB plus 1% starch (B), SLB plus 0.2% starch (C), and SLB plus 3% Tween 80 (D) were inoculated with a culture of *P. jensenii* and incubated anaerobically at 30°C. The growth was monitored, and the cells and supernatant of the culture were measured for hemolytic activity. Symbols: ◇, growth (optical density at 600 nm); ■, hemolytic activity of the cells; ▲, hemolytic activity of the culture supernatants.

the hemolytic activity was measured throughout growth (Fig. 1B to D). The highest hemolytic titer was achieved with 1% starch in the growth medium, and the starch acted as a better stabilizer than Tween 80. Although it was also possible to extract the hemolytic activity and pigment directly from the cells with the extractor solution, this method did not produce hemolytic titers as high as those obtained by growing the cells with starch.

The hemolytic starch extracts from *P. jensenii* had a strong orange color and displayed absorption peaks of 463 nm, 492 nm, and 524 nm in the UV-VIS spectrometer (Fig. 2A). These peaks were absent in the absorption spectra of the supernatants of cultures grown without starch.

Characteristics of the hemolytic extract. The hemolytic activity of *P. jensenii* was dose, time, and temperature dependent in a manner similar to that of *S. agalactiae* (18, 20). The activity showed an initial lag followed by a sigmoidal decline in turbidity as a function of time. With increases in temperature, the lag period was shortened and the rate of the hemolytic activity increased (Fig. 3). The lysis of erythrocytes was also dependent upon the concentration of hemolysin. The length of the prelytic lag period shortened, and the rate of hemolysis increased at higher concentrations of hemolysin (Fig. 4).

Inactivation experiments with protease, alpha-amylase, and heat were performed. Incubation of extract with proteinase K reduced the hemolytic activity by approximately 50%, which indicates that a protein was involved in the activity. The hemolysin was sensitive to heat, and after 10 min at 60°C the activity was reduced by 99.5%. Alpha-amylase also reduced the activity by 86%, probably by destabilizing the carrier function of the starch. When the hemolytic extract was digested with alpha-amylase, a color change from orange to yellow was observed; the same effect was observed when the extract was heated. The simultaneous loss of hemolytic activity and a change in the color of the hemolytic extract indicate a physical link between the hemolytic activity and pigment.

Osmotic protection experiment with PEG. Macromolecules such as PEG can inhibit the osmotic lysis of pore-forming hemolysins by compensating for the osmotic imbalance (22). Assays with PEGs of different molecular weights affected the hemolysin from *P. jensenii*. Whereas PEG 1500 caused a delay in hemolysis, the presence of PEGs with higher molecular masses completely inhibited hemolysis (Fig. 5). These results indicate that the hemolytic effect is caused by a pore-forming mechanism. Given that the hydrodynamic radii of PEG 1500 and PEG 3000 are 1.1 and 1.4 nm, respectively (12) the experiment suggests that the pore radius is between these values. For comparison, perfringolysin O from *Clostridium perfringens*

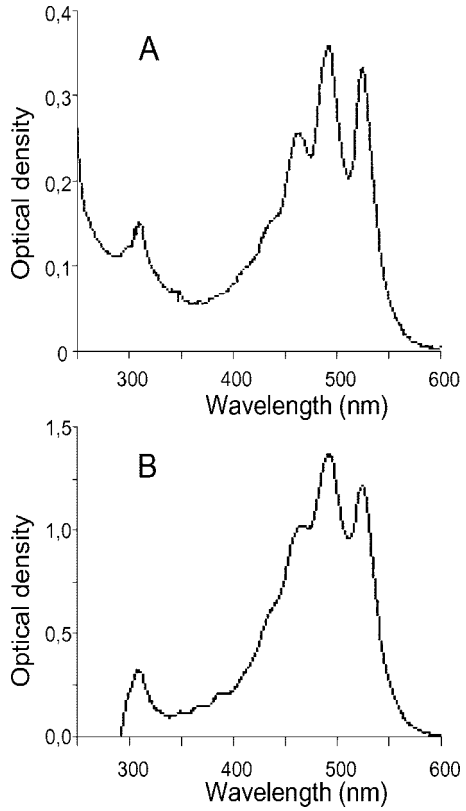

FIG. 2. UV-VIS spectra of pigment extracts. (A) Absorption spectrum of hemolytic extract; (B) absorption spectrum of isolated pigment dissolved in DMSO-0.1% TFA.

makes pores with a radius of 3.5 nm (5). To our knowledge the size of the pores caused by *S. agalactiae* hemolysin has not been determined.

Partial purification of hemolytic activity. Efforts were made to purify the hemolytic activities from culture supernatants. To reduce the amount of starch in the extract, cultures cultivated with 0.2% starch or 20% extractor solution were used. The addition of methanol precipitated the hemolytic activity along with the starch and produced an orange-colored starch pellet. This pellet was dissolved in 5 mM Tris-HCl (pH 8.0) and applied to a HiPrep 16/10 Q XL column. Most of the activity bound to the column, and up to half of the activity could be eluted with 0.3 M NaCl. The rest of the starch-hemolysin-pigment complex was firmly attached to the column and could be completely eluted only by the addition of 1 M NaOH. The alkaline treatment released active material in fractions with a pH close to neutral. The fractions containing hemolytic activity contained an orange color whose intensity coincided with the hemolytic titers of the fractions. Under no circumstances was it possible to obtain any hemolytic fractions without the pigment,

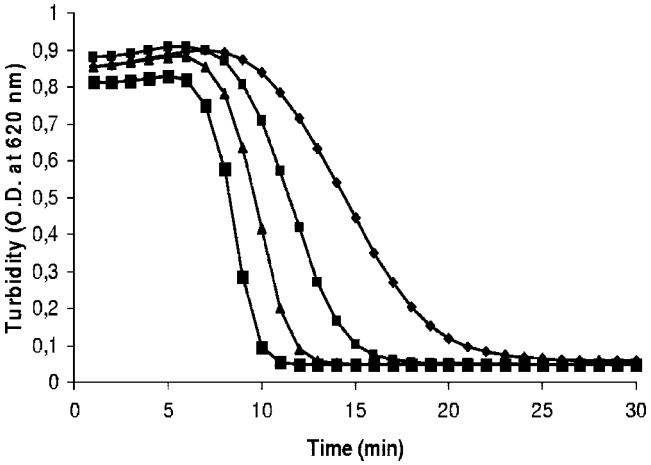

FIG. 3. Effect of temperature on hemolytic activity. Horse erythrocytes (1%) were incubated with hemolytic extract (128 HU), and the hemolysis was assayed by determining the decrease in turbidity. Symbols: ◆, 25°C; small ■, 30°C; ▲, 37°C; large ■, 42°C.

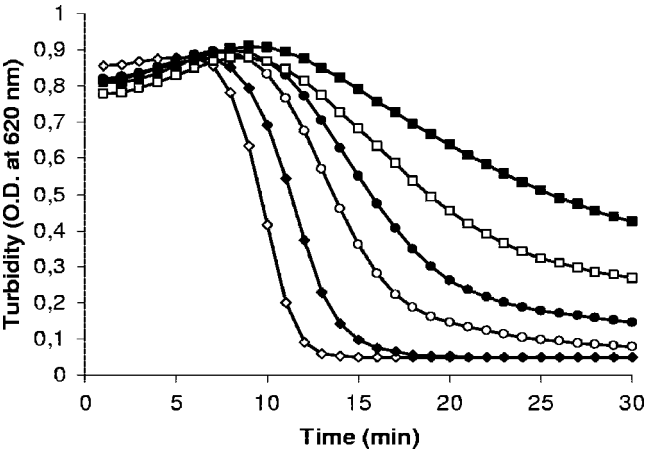

FIG. 4. Effect of hemolysin concentration on hemolysis. A suspension of horse erythrocytes (1%) was incubated with different concentrations of hemolytic extract and incubated at 37°C. Hemolysis was assayed by the decrease in turbidity. Symbols: ■, 10 HU/ml; □, 20 HU/ml; ●, 40 HU/ml; ○, 80 HU/ml; ◆, 320 HU/ml; ◇, 1,280 HU/ml.

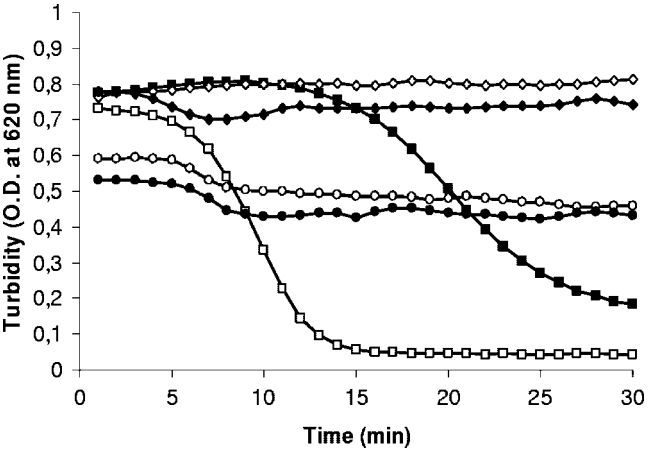

FIG. 5. Effect of osmotic protectants on hemolysis. Horse erythrocyte (1%) suspensions containing PEGs of different molecular weights were incubated with hemolytic extract (36 HU) at 37°C. Hemolysis was assayed by determining the decrease in turbidity. Symbols: □, without PEG; ■, PEG 1500; ◇, PEG 3000; ◆, PEG 6000; ○, PEG 8000; ●, PEG 8000 without hemolytic extract. Since an erythrocyte suspension with PEG 8000 gave a slight reduction in the reading of the turbidity, the control is also displayed in the figure.

which strengthens the idea that the pigment is associated with the hemolytic activity. The fractions also still contained starch in association with the hemolytic activity and pigment. The active fractions were precipitated with methanol and subjected to phenol extraction, but a complete removal of starch was difficult. This treatment destroyed the activity, and when the sample was analyzed by SDS-polyacrylamide gel electrophoresis, several protein bands could be seen. The requirement for starch to stabilize the activity severely hampered any further purification. It was not possible to remove the starch and still conserve any hemolytic activity. Attempts were also made to purify the hemolytic activity from the Tween 80 extracts, but the activity was too unstable for our attempts to be successful.

Purification and identification of pigment (by MS and NMR). The presence of the orange color in all the fractions with hemolytic activity suggests a physical linkage between the two properties. The absorption spectrum of the hemolytic extract revealed absorption peaks typical of a carotenoid. However, the pigment could not be extracted from the cells with methanol or methanol-acetone mixtures commonly used for carotenoids. The pigment could be extracted from the cells by washing the cells with 20 mM sodium phosphate buffer (pH 7.0). The pigment lost its attachment to the cell surface, and the behavior is suggestive of a hydrophobic interaction of the pigment to compounds on the cell surface. The pigment did not stay in solution but precipitated rapidly. This aggregated form of pigment was not soluble in any solvents (methanol, acetone, chloroform, phenol, water, SDS, urea, guanidine hydrochloride, DMSO, or even the extraction solution containing starch) and gave a single absorption peak of 415 nm in the UV-VIS spectrum. Rosa-Fraile et al. (26) showed that DMSO-0.1% TFA could dissolve the pigment from *S. agalactiae*; this was indeed also the case for the pigment from *P. jensenii*. The dissolved pigment gave a carotene-like spectrum with absorption peaks of 464 nm, 490 nm, and 522 nm, equivalent to the absorption peaks of the hemolytic extract (Fig. 2B).

The MS analysis of the dissolved pigment showed M + H ions at *m/z* 677.469 and M + Na ions at *m/z* 699.441, in accordance with the molecular mass of 676 of granadaene (see Fig. S1A in the supplemental material). However, additional peaks of M + H ions at *m/z* 719.476 and M + Na ions at *m/z* 741.470, in agreement with a molecular mass of 718.4, were observed in the spectrum, which could account for an acetylated variant of the pigment, but this was not detected by NMR. Hydrolysis of the acetyl group could occur during the purification procedure, so the possibility of the presence of an acetylated variant of the pigment cannot be excluded.

The NMR chemical shifts were in agreement with those published for granadaene (see Table S1 in the supplemental material). The position of the glycosidic moiety was confirmed by the long-range HMBC correlation between H-1″ and C-27. The HMBC correlation between H-2′ and C-1 across the amide nitrogen could not be observed, nor could any other HMBC correlations with the two carbonyl carbons. Their chemical shifts could therefore not be determined.

Regarding the stereochemical aspects of granadaene, the all-*E* (all-*trans*) configuration was assigned to the double bonds based on the UV-VIS spectrum (maximum λ and fine structure) of the starch extract (Fig. 2A) and the $^{1}J_{H,H}$ coupling constants of C-2 and -3, C-4 and -5, and C-24 and -25. In solution, especially in the presence of acid, isomerization of the double bonds leads to a hypsochromic shift and loss of fine structure over time. The structure of granadaene is depicted in Fig. S1B in the supplemental material.

In *S. agalactiae*, the level of hemolytic activity correlates with the amount of the polyene pigment granadaene (30). Molecular methods such as transposon mutagenesis and complementation studies have verified the close genetic linkage between the hemolysin and granadaene in *S. agalactiae* (25, 28). Despite this, the hemolysin has never been isolated because of the instability and the requirement for starch to preserve the activity. The gene *cylE*, which is a part of the operon for the biosynthesis of granadaene from *S. agalactiae*, is proposed to encode the hemolysin (25, 28). Speculations as to whether CylE is the hemolysin itself or whether it contributes to the synthesis of the hemolysin have been made (8). Furthermore, a complex could exist between a hemolytic protein and the polyene pigment in situations where the pigment aids in the hemolytic activity (14).

It has not been possible to purify any hemolytic protein from *P. jensenii* or *S. agalactiae*. What we have found in common is the polyene pigment consistently associated with the hemolytic activities. The sensitivity to heat and proteases indicates the involvement of a protein in hemolysis. Nevertheless, the pigment is a polyene, and it is known that some polyenes, like the antibiotic amphotericin B produced by *Streptomyces nodosus*, are lytic to erythrocytes in high concentrations (2, 11). The polyene part of amphotericin B is believed to be important for the function of the polyene, since it interacts with sterols in both fungal and mammalian membranes (33). It is tempting to speculate that the polyene part of granadaene interacts with sterols, like cholesterol, in mammalian cell membranes either by itself or in concordance with a protein. However, purified pigment from *P. jensenii* dissolved in DMSO-0.1% TFA showed no hemolytic activity when spotted onto blood agar.

Propionibacterium jensenii is not related to *S. agalactiae*; hence, the production of an identical pigment and a similar hemolytic system is surprising. A strong link between pigment and hemolysis exists in both bacteria. The hemolytic system in *S. agalactiae* is well studied, but the exact nature of the hemolytic activity is still not clear. Further studies of *P. jensenii* will reveal any similarities at the genetic level. The β-hemolysin from *S. agalactiae* affects a broad range of host cells (15, 21, 31) and is considered to be an important virulence factor in the pathogenesis of invasive infections (9, 20). The strong similarities, concerning the hemolytic system, with *S. agalactiae* raise questions about the absolute safety of *P. jensenii*.

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