Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Structural studies of *Streptococcus agalactiae* hyaluronate lyase

Mark J. Jedrzejas* and Laurent Chantalat

Department of Microbiology, 933 19th Street South, 545 CHSB-19, University of Alabama at Birmingham, Birmingham, Alabama 35294-2041, USA

Correspondence e-mail: jedrzejas@uab.edu

Received 11 October 1999
Accepted 13 January 2000

The bacteria *Streptococcus agalactiae*, part of normal human flora, produce an enzyme, hyaluronate lyase, which appears to contribute to the invasive capacity of this pathogen by degrading hyaluronan and chondroitin sulfates of the extracellular matrix of host tissues. The native enzyme, the product of the $hylB_{3502}$ allele, has a molecular mass of 111 kDa but undergoes an autocatalytic conversion to a smaller enzymatically active 92 kDa form. To initiate the determination of the catalytic mechanism of action of these enzymes, the 111 and 92 kDa enzymes were crystallized by a vapor-diffusion method using polyethylene glycol monomethyl ether 5000 and potassium thiocyanate as precipitating agents. The 111 kDa enzyme crystals are of poor quality and diffract X-rays to a very low resolution. However, the crystals of the truncated 92 kDa enzyme diffract X-rays to 2.1 Å resolution. The crystal symmetry is $C222_1$ and the unit-cell parameters are $a = 51.69$, $b = 157.03$, $c = 239.20$ Å ($\alpha = \beta = \gamma = 90^\circ$). The V_m of 2.64 Å^3 Da^{-1} is consistent with the presence of one molecule of hyaluronate lyase in the asymmetric unit and a crystal solvent content of 53%. An isomorphous ethylmercuricthiosalicylic acid heavy-atom derivative diffraction data set has been obtained in order to solve the structure.

1. Introduction

S. agalactiae (group B streptococcus; GBS) bacterial pathogen is part of the normal human flora (Dillon *et al.*, 1987). The mechanisms of *S. agalactiae* invasion of human host tissues and the penetration of the physical defenses of the host are, however, unknown. A diverse array of potent bacterial products is probably involved in host injury. One of these possible harmful products is hyaluronate lyase enzyme. *S. agalactiae* hyaluronate lyase is associated with full bacterial virulence of this organism (Milligan *et al.*, 1978; Musser *et al.*, 1989). In cultures of *S. agalactiae* and *S. pneumoniae*, the enzyme is found both in the culture and in the cell-associated fractions. This may suggest that at least part of the enzyme is released by the pathogen to surrounding host tissues during infection in order to facilitate bacterial invasion (Berry *et al.*, 1994).

Hyaluronate lyase is a major surface protein of *S. agalactiae* bacteria and is capable of breaking down two components of the extracellular matrix of tissues, hyaluronan and certain chondroitin sulfates (Laurent & Fraser, 1992; Pritchard *et al.*, 1994). It appears to contribute to the invasive capacity of the bacteria. Hyaluronate lyase facilitation of tissue invasion by breaking down the extracellular matrix (ECM) components causes increased tissue permeability and appears to play a role in wound infections, pneumonia and other sepsis. Unfortunately, the exact mechanism of how the enzyme facilitates the bacteria's ability to penetrate the host's physical defenses and subsequently spread to its tissues is still poorly understood (Mufson, 1990; Boulnois, 1992; Busse, 1991).

The primary substrate of hyaluronate lyase is hyaluronan, a ubiquitous and important component of the ECM of vertebrates, the main functions of which are joint lubrication and shock absorption. Hyaluronan is a polymer composed of repeating units of D-glucuronic acid-(1-β-3)-*N*-acetyl-D-glucosamine-(1-β-4). It is detectable in every studied tissue and fluid in higher animals and in humans (Laurent & Fraser, 1992). In addition to hyaluronan's role as a structural component, there is growing evidence that hyaluronan has many other functions in the host defense mechanisms. It has been implicated in processes including cell migration and differentiation, and growth and metastasis of tumor cells (Gibson *et al.*, 1991; Teti *et al.*, 1993; Williams *et al.*, 1993). The cell-surface receptor for hyaluronan is CD44, which is present on many different cell types and seems to be important in various different steps in the normal immune response (Haynes *et al.*, 1989). Hyaluronan levels are finely controlled in

Table 1
Statistics of the X-ray diffraction data for the 92 kDa *S. agalactiae* hyaluronate lyase.

Synchrotron X-ray diffraction data were acquired using a Brandeis-4 CCD detector at Brookhaven National Laboratory using beamline x12c and X-ray radiation at $\lambda = 0.95$ Å.

Resolution shell (Å)	Number of unique reflections	$I/\sigma(I)$	R_{sym}† (%)	Completeness (%)
50.00–4.52	5788	33.9	0.034	97.5
4.52–3.59	5600	32.6	0.040	98.4
3.59–3.14	5625	30.2	0.046	99.3
3.14–2.85	5571	27.3	0.062	99.5
2.85–2.65	5626	22.2	0.079	99.4
2.65–2.49	5589	16.8	0.099	99.6
2.49–2.37	5576	14.0	0.126	99.8
2.37–2.26	5587	10.6	0.147	98.1
2.26–2.18	5396	8.2	0.159	94.5
2.18–2.10	4522	6.3	0.177	77.2
Total	54880	23.2	0.053	97.0

† $R_{sym} = \sum|I - \langle I\rangle|/\sum I$, where I is the intensity for an observation of a multiply observed reflection.

order to regulate the rate of hyaluronan biosynthesis and degradation (Fraser & Laurent, 1989; Sampson *et al.*, 1992).

Hyaluronate lyase has been reported to cleave glycosidic bonds in hyaluronan (hyaluronic acid polymer) by the β-elimination process, which involves the introduction of an unsaturated double bond in the polysaccharide end product (Pritchard & Lin, 1993). This mechanism is different from other hyaluronidases, such as animal hyaluronidases, which are hydrolases. The final end product of hyaluronan degradation by the *S. agalactiae* and *S. pneumoniae* enzymes are disaccharide units of hyaluronan, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose (M. J. Jedrzejas, unpublished results; Pritchard *et al.*, 1994).

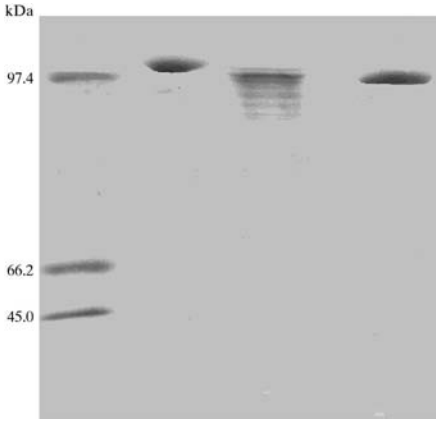

Figure 1
SDS–PAGE of hyaluronate lyase was carried out under reducing conditions in a 8% polyacrylamide gel using the buffer described by Laemmli (1970). The gels were stained with Coomassie Blue. The molecular-weight standards are indicated on the left. The first lane is the purified 111 kDa enzyme, the second lane contains the same enzyme incubated for 2 weeks at 277 K in the absence of EDTA and the third lane shows the band for the 92 kDa eluant from the Superdex 200 gel-filtration column. The protein load for each lane was approximately 5 µg.

The sequences of genes for the two streptococcal hyaluronate lyases, *S. agalactiae* and *S. pneumoniae*, have been determined to be 68% homologous, suggesting their functional, structural and evolutionary similarities (Berry *et al.*, 1994; Jedrzejas, Mewbourne *et al.*, 1998). Also, recently reported sequences for hyaluronate lyases from *Staphylococcus aureus* (Hynes & Hancock, 1995; Steiner & Cruce, 1992) and *Propionibacterium acnes* (Steiner & Cruce, 1992; GeneBank accession No. U27583) show high amino-acid similarity to the *S. agalactiae* enzyme.

The gene for *S. agalactiae* hyaluronate lyase ($hylB_{3502}$) has been cloned, sequenced, expressed in *Escherichia coli* and the enzyme purified by cation-exchange chromatography as previously reported by Lin *et al.* (1994). In the current study, we report the degradative properties of this enzyme's preparations as well as its crystallization and initial X-ray diffraction analysis.

2. Experimental

2.1. Analytical methods

Recombinant *S. agalactiae* hyaluronate lyase, the product of the $hylB_{3502}$ allele, was provided by Dr David Pritchard. The protein sample was dialyzed against 10 m*M* HEPES buffer pH 8.0, 150 m*M* NaCl, 5 m*M* EDTA. The sample was concentrated to 10 mg ml^{-1}, which was found to be appropriate for crystallization experiments using a Centricon 30 ultrafiltration device (Amicon). Electrophoresis was carried out under reducing conditions in a 8% polyacrylamide gel using the buffer system described by Laemmli (1970) and a Mini Protein II gel system from Bio-Rad. The gels were stained with Coomassie Blue (Fig. 1). Isoelectric focusing experiments were carried out at room temperature using the PhastSystem (Amersham Pharmacia Biotech) with high-pH-range gels (pH 6.0–9.0).

Quantitative assays for hyaluronate lyase activity were carried out as reported previously (Jedrzejas, Mewbourne *et al.*, 1998; Pritchard *et al.*, 1994). One enzyme unit of hyaluronate lyase activity was defined as the amount of enzyme that catalyzes the release of 1 µmol of the unsaturated disaccharide 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-glucose from hyaluronan per minute in 50 m*M* ammonium acetate buffer pH 6.0, 5 m*M* $CaCl_2$. A millimolar absorption coefficient of 5.5 was used in all calculations (Yamagata *et al.*, 1968). The assay was performed at room temperature.

2.2. Enzyme conversion and gel-filtration chromatography

The 111 kDa *S. agalactiae* hyaluronate lyase was converted to the 92 kDa form by the addition of excess $CaCl_2$ (10 m*M*) to the protein sample followed by incubation at 277 K for at least 14 d, during which the conversion process took place. The enzyme conversion was traced by gel electrophoresis. The truncated form (92 kDa) was applied to a 30 × 1.0 cm Superdex 200 gel-filtration column (Amersham Pharmacia Biotech) equilibrated with 10 m*M* HEPES buffer, 2 m*M* EDTA pH 8.0. The eluted fractions corresponding to the 92 kDa enzyme form were pooled together. The concentration of the enzyme was adjusted for the crystallization trials to approximately 10 mg ml^{-1} (Pace *et al.*, 1995) using Centricon 10 and Centriplus 10 devices (Amicon).

2.3. Crystallization

Since *S. agalactiae* hyaluronate lyase is a basic protein (pI = 8.8), the method developed by Riès-Kautt & Ducruix (1991) was used to crystallize this protein by ion pairing. Using this methodology, a basic protein at acidic pH is most easily precipitated by anions in the series $SCN^- > Cl^- > HCO_3^- >$ citrate^{2-} > $CH_3COO^- \simeq PO_4^{2-} > SO_4^{2-}$ (Hofmeister, 1988; Riès-Kautt & Ducruix, 1989, 1991). In addition, the sparse-matrix screen method utilizing commercially available screens from Hampton Research, Inc. was used in determination of the nucleation and crystallization conditions.

The vapor-diffusion method (McPherson, 1999) was used to obtain hyaluronate lyase crystals with polyethylene glycol monomethyl ether 5000 (PEG-MME 5000) and potassium thiocyanate (KSCN) as precipitating agents at room temperature. The reservoir solution contained 100 m*M* cacodylate buffer pH 6.0, 30 m*M* KSCN and various amounts of PEG-MME 5000. The crystallization hanging drops contained 1–5 µl of the protein solution and 1–5 µl of the reservoir solution and were equilibrated against 1 ml of reservoir solution.

2.4. X-ray diffraction and data collection

Initial diffraction studies of the crystals of the 92 kDa form of the enzyme were performed at room temperature. However,

Table 2
Statistics of the heavy-atom X-ray diffraction data.

The heavy-atom data for the EMTS† derivative were acquired using an R-AXIS II image-plate detector, a Rigaku RU-200 rotating-anode generator and Cu $K\alpha$ radiation. Numbers in parentheses correspond to the highest resolution shell.

	Native	Derivative EMTSA†
Resolution limit (Å)	2.1	2.9
No. of reflections measured	197090	40766
No. of unique reflections	54880	16149
Completeness (%)	97.0 (77.2)	75.2 (34.5)
$I/\sigma(I)$	23.2 (6.3)	9.5 (2.4)
R_{sym} (%)	5.3 (17.7)	7.3 (19.7)
R_{iso}‡ (%)	Not applicable	18.0

† EMTSA – ethylmercuricthiosalicylic acid, 1 m*M* soak for 12 h. ‡ $R_{iso} = \sum_h |F_P(h) - F_{PH}(h)| / \sum F_P(h)$, where $F_P(h)$ and $F_{PH}(h)$ are native and scaled derivative structure factors of the reflection h.

such crystals decayed rapidly in the X-ray beam. To minimize the decay, crystals were cryoprotected by immersing them for a few seconds in a cryoprotectant solution containing 22% glycerol, 30% PEG-MME 5000, 30 m*M* KSCN, 50 m*M* sodium cacodylate buffer pH 6.0 and were then frozen in a nitrogen flow at 103 K using a Cryostream Cooler low-temperature device (Oxford Cryosystems). Under such conditions, the crystals showed essentially no decay in the resolution of diffraction. The heavy-atom derivative crystals were prepared by soaking the native crystals in solutions of appropriate heavy-atom compounds in 30% PEG-MME 5000, 30 m*M* KSCN, 50 m*M* sodium cacodylate buffer pH 6.0 for various lengths of time. The heavy-atom compounds used were ethylmercuricthiosalicylic acid (EMTS), potassium tetrachloroplatinate (II), mercury (II) acetate and barium chloride.

The native data set was collected using synchrotron X-ray radiation at 0.95 Å (Brookhaven National Laboratory, National Synchrotron Light Source x12c beamline) and a Brandeis-4 CCD detector. The data were indexed, integrated and scaled with the *HKL* package (Otwinowski & Minor, 1997). The heavy-atom derivative diffraction data were collected on cryo-frozen crystals using a Cu $K\alpha$ rotating-anode X-ray source mounted on a Rigaku RU-200 generator and an R-AXIS II image-plate detector (Molecular Structure Corporation).

3. Results and discussion

3.1. Conversion of the native enzyme to a 92 kDa form

It was initially observed that *S. agalactiae* hyaluronate lyase, the product of the $hylB_{3502}$ allele, isolated from *E. coli* and stored in neutral solution at 277 K or at room temperature for several weeks, converted from a 111 kDa form to a 92 kDa form without a significant change in the enzyme activity (Figs. 1 and 2). The specific activities of the 111 kDa and the truncated enzyme were 3680 and 3571 U mg^{-1}, respectively (Fig. 2). Highly purified preparations of the recombinant enzyme exhibited similar conversion with storage and during crystallization trials, suggesting that the process might be autocatalytic. A known inhibitor of proteolytic enzymes, phenylmethanesulfonyl fluoride (PMSF), did not inhibit the conversion. However, the presence of excess EDTA (2 m*M*) in the protein sample was found to completely prevent the conversion to the lower molecular mass form. *S. agalactiae* hyaluronate lyase has been reported to require calcium for optimal activity and its inhibition by EDTA is likely to be based on EDTA's ability to bind divalent cations such as calcium. However, degradation by proteases requiring calcium for activity but not inhibited by PMSF cannot be discarded.

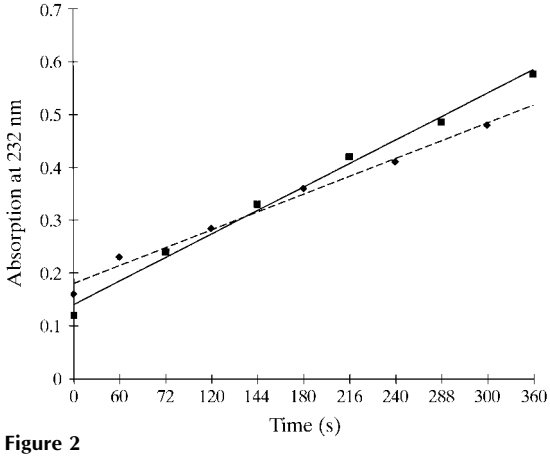

Figure 2
Activity of the recombinant 92 and 111 kDa *S. agalactiae* hyaluronate lyase enzymes was followed by the rate of increase in absorbance at 232 nm (corresponding to an increase in enzyme activity) after addition of 5.0 µg of the enzyme to 1.0 ml of 0.2 mg ml^{-1} solution of human umbilical cord hyaluronan at room temperature (squares, native enzyme; diamonds, truncated enzyme).

The 111 kDa *S. agalactiae* hyaluronate lyase was purified in the presence of 2 m*M* EDTA using cation-exchange chromatography as reported by Lin *et al.* (1994). It was then converted to the 92 kDa form as described in §2. In order to recover the 92 kDa form of hyaluronate lyase, the enzyme solution was further purified by gel-filtration chromatography. Polyacrylamide-gel electrophoresis of both forms of the enzyme conducted under denaturing conditions resulted in single bands of apparent molecular mass 92 and 111 kDa (Fig. 1). The isoelectric point of both forms was determined as described in §2 to be ∼9, which agrees with the values calculated based on sequence of both forms of the enzyme. Both enzymes were shown to have a single isoelectric focusing isoform.

The amino-acid sequence of the N-terminus of the purified 92 kDa enzyme was determined. The sequence obtained for the first six residues was SEHPQP. This sequence corresponds to residues 171–176 of the predicted amino-acid sequence for the 111 kDa protein. Additionally, it is likely that the 111 kDa form of the *S. agalactiae* enzyme, the product of $hylB_{3502}$ allele, is also a truncated but active form rather than a full-length mature enzyme. The undegraded mature form of this enzyme seems to have an approximate mass of 118 kDa (Gase *et al.*, 1998). A similar behavior was reported for the *S. pneumoniae* hyaluronate lyase, in which the truncated 89 kDa form of the protein lacks the first N-terminal 163 residues from the 107 kDa enzyme (Berry *et al.*, 1994; Jedrzejas, Chantalat *et al.*, 1998; Jedrzejas, Mewbourne *et al.*, 1998). The gene for *S. agalactiae* hyaluronate lyase ($hylB_{3502}$) was found to have 50.7% amino-acid identity to the hyaluronate lyase from *S. pneumoniae* (Berry *et al.*, 1994). The 92 kDa form of the *S. agalactiae* enzyme (lacking the first N-terminal 170 residues of the 111 kDa form) and the 89 kDa form of the *S. pneumoniae* enzyme (lacking the first N-terminal 163 residues of the 107 kDa enzyme) retain high specific activities comparable with those of the native enzymes (Berry *et al.*, 1994; Jedrzejas, Mewbourne *et al.*, 1998). The specific activity of the truncated *S. agalactiae* enzyme at room temperature is only 3% lower than that of the 111 kDa enzyme (Fig. 2). Clearly, for these two streptococcal hyaluronate lyase enzymes, the N-terminal region is not essential for activity.

3.2. Crystallization and X-ray diffraction

The 92 kDa and the 111 kDa enzymes were crystallized by a vapor-diffusion method using polyethylene glycol monomethyl ether 5000 (PEG-MME 5000) as precipitating agent (McPherson, 1999). The native enzyme crystals grown at pH 6.0–8.0 were of poor quality and diffracted X-rays to a very low resolution. Trials to improve their quality proved unsuccessful.

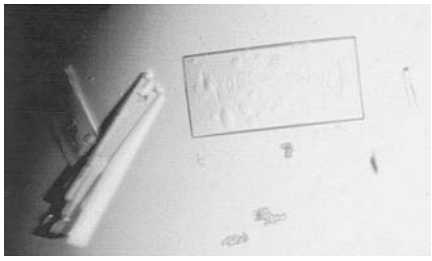

Figure 3
Crystals of 92 kDa recombinant hyaluronate lyase enzyme grew in one week using the hanging-drop vapor-diffusion method at 195 K with PEG-MME 5000 and KSCN as precipitating agents at pH 6.0. Their size is 400 × 200 × 70 µm and they diffract X-rays to 2.1 Å resolution.

Single crystals of the 92 kDa form of the enzyme were of much better quality. They were obtained following the method for crystallizing basic proteins introduced by Riès-Kautt & Ducruix (1991). Crystallization experiments of the truncated 92 kDa form were set up with potassium thiocyanate (KSCN) as the main precipitating agent, with varying concentrations of KSCN (30–300 m*M*) and with pH in the range 4–7. As expected, higher protein precipitation occurred at lower pH, where the enzyme has an increased total positive charge and therefore there are more positive protein charges to shield by the SCN^- ions. However, no crystals were observed. Based on our previous crystallization experiments, it was noted that using PEG-MME 5000 as a precipitating agent produced round irregular crystals of the enzyme. However, a combination of both precipitating agents was needed in order to grow crystals of good quality. The first micro-crystals were observed as sharp needles at pH 6.0 using a 100 m*M* sodium cacodylate buffer, 50 m*M* KSCN, 19% PEG-MME 5000. To further improve the crystals, the concentration of KSCN was varied from 10 to 200 m*M*. At 30 m*M* KSCN, only a few good-quality crystals could be grown. At KSCN concentrations lower than 25 m*M* no crystals were observed, while at overly high concentrations too many nucleation sites were created, leading to the formation of a large number of crystals having a long needle-like shape. KSCN was essential for the crystallization of well formed and well diffracting crystals. It is believed that SCN^- ions shield charges on the enzyme molecules, allowing them to interact closely in the crystal matrix.

The best crystals of the truncated enzyme have a rectangular plate shape and grew to approximate dimensions of 400 × 200 × 70 µm within one week (Fig. 3). Diffraction to 2.1 Å resolution was observed using a synchrotron X-ray source and a CCD detector. Unit-cell parameters were derived by an autoindexing procedure of the *DENZO* program (Otwinowski & Minor, 1997). The space group of these crystals was determined to be orthorhombic $C222_1$, with unit-cell parameters a = 51.69, b = 157.03, c = 239.20 Å. The crystal volume per unit of protein molecular weight value, V_m, of 2.64 $Å^3\ Da^{-1}$ is consistent with one hyaluronate lyase molecule in the asymmetric unit and a solvent content of 53.4% (Matthews, 1968). The native X-ray diffraction data set was acquired using synchrotron radiation and the data were processed and scaled with the *HKL* package (Otwinowski & Minor, 1997; Table 1). The search for suitable heavy-atom derivatives to solve the structure by multiple isomorphous replacement methods is under way. The initial isomorphous heavy-atom derivative using ethylmercuricthiosalicylic acid (EMTS) has been identified and appropriate derivative X-ray diffraction data have been collected and compared with the native diffraction data (Table 2). In summary, the flash-freezing conditions together with synchrotron radiation facilitated the collection of high-quality native diffraction data. These conditions will be used to obtain heavy-atom derivative data sets of equal quality. This will allow the determination of the *S. agalactiae* hyaluronate lyase structure solution at high resolution.

The authors are grateful to Dr David G. Pritchard, University of Alabama at Birmingham, for providing the protein and the clone of the *S. agalactiae* hyaluronate lyase. The peptide-sequence analysis was performed at the UAB Comprehensive Cancer Center shared facility. Diffraction data for this study were collected at Brookhaven National Laboratory in the Biology Department single-crystal diffraction facility at beamline x12c in the National Synchrotron Light Source. This facility is supported by the United States Department of Energy Offices of Health and Environmental Research and of Basic Energy Sciences under prime contract DE-AC02-98CH10886, by the National Science Foundation and by National Institutes of Health (NIH) Grant 1P41 RR12408-01A1. This work was supported by NIH grant R01 AI44079 (to MJJ).

References

Berry, A. M., Lock, R. A., Thomas, S. M., Rajan, D. P., Hansman, D. & Paton, J. C. (1994). *Infect. Immun.* **62**, 1101–1108.
Boulnois, G. J. (1992). *J. Gen. Microbiol.* **138**, 249–259.
Busse, W. (1991). *Rev. Infect. Dis.* **13**, S477–S485.
Dillon, H. C., Khare, S. Jr & Gray, B. M. (1987). *J. Pediatr.* **110**, 31–36.
Fraser, J. R. E. & Laurent, T. C. (1989). *Ciba Foundation Symposium 143. Turnover and Metabolism of Hyaluronan*, edited by D. Evered and J. Whelan, pp. 42–59. Chichester: John Wiley & Sons Ltd.
Gase, K., Ozegowski, J. & Malke, H. (1998). *Biochim. Biophys. Acta*, **1398**, 86–98.
Gibson, R. L., Redding, G. J., Henderson, W. R. & Truog, W. E. (1991). *Am. Rev. Respir. Dis.* **143**, 598–604.
Haynes, B. F., Telen, M. J., Hale, L. P. & Denning, S. M. (1989). *Immunol. Today*, **10**, 423–428.
Hofmeister, F. (1988). *Arch. Exp. Pathol. Pharm.* **24**, 247–260.
Hynes, W. L. & Hancock, L. (1995). *Infect. Immun.* **63**, 3015–3020.
Jedrzejas, M. J., Chantalat, L. & Mewbourne, R. B. (1998). *J. Struct. Biol.* **121**, 73–75.
Jedrzejas, M. J., Mewbourne, R. B., Chantalat, L. & McPherson, D. (1998). *Protein Expr. Purif.* **13**, 83–89.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Laurent, T. C. & Fraser, J. R. E. (1992). *FASEB J.* **6**, 2397–2402.
Lin, B., Hollingshead, S. K., Coligan, J. E., Egan, M. L., Baker, J. R. & Pritchard, D. G. (1994). *J. Biol. Chem.* **269**, 30113–30116.
McPherson, A. (1999). *Crystallization of Biological Macromolecules.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Milligan, T. W., Baker, C. J., Straus, D. C. & Mattingly, S. J. (1978). *Infect. Immun.* **21**, 738–746.
Mufson, M. A. (1990). *Principles and Practice of Infectious Diseases. Streptococcus Pneumoniae*, edited by G. L. Mandell, R. G. Douglas Jr and J. E. Bennett, pp. 1539–1550. New York: Churchill Levingstone.
Musser, J. M., Mattingly, S. J., Quentin, R., Goudeau, A. & Selander, R. K. (1989). *Proc. Natl Acad. Sci.* **86**, 4731–4735.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Pace, C. N., Vajdos, F., Fee, L., Grimsley, G. & Gray, T. (1995). *Protein Sci.* **4**, 411–2423.
Pritchard, D. G. & Lin, B. (1993). *Infect. Immun.* **61**, 3234–3239.
Pritchard, D. G., Lin, B., Willingham, T. R. & Baker, J. R. (1994). *Arch. Biochem. Biophys.* **315**, 431–437.
Riès-Kautt, M. M. & Ducruix, A. F. (1989). *J. Biol. Chem.* **264**, 745–748.
Riès-Kautt, M. M. & Ducruix, A. F. (1991). *J. Cryst. Growth*, **110**, 20–25.
Sampson, P. M., Rochester, C. L., Freundlich, B. & Elias, J. A. (1992). *J. Clin. Invest.* **90**, 1492–1503.
Steiner, B. & Cruce, D. (1992). *Anal. Biochem.* **200**, 405–410.
Teti, G., Mancuso, G. & Tomasello, F. (1993). *Infect. Immun.* **61**, 227–235.
Williams, P. A., Bohnsack, J. F., Augustine, N. H., Rubens, C. E. & Hill, H. R. (1993). *J. Pediatr.* **123**, 292–300.
Yamagata, T., Saito, H., Habuchi, O. & Suzuki, S. (1968). *J. Biol. Chem.* **243**, 1523–1535.