PROTEINS: Structure, Function, and Genetics 28:580–585 (1997)

Crystals of Cytochrome c-553 From *Bacillus pasteurii* Show Diffraction to 0.97 Å Resolution

Stefano Benini,[1] Stefano Ciurli,[1]* Wojciech R. Rypniewski,[2] and Keith S. Wilson[3]
[1]*Institute of Agricultural Chemistry, University of Bologna, Bologna, Italy*
[2]*European Molecular Biology Laboratory, Hamburg, Germany*
[3]*Department of Chemistry, University of York, Heslington, York, United Kingdom*

ABSTRACT **We report here the purification and characterization of a *c*-type cytochrome present in the soluble fraction of the gram-positive, alkaliphilic, and highly ureolytic soil bacterium *Bacillus pasteurii*. The cytochrome is acidic (pI = 3.3), has a molecular mass of 9.5 kDa, and appears to dimerize in 150 mM ionic strength solution. The electronic spectrum is typical of a low-spin hexa-coordinated heme iron. Crystals of the protein in the oxidized state were grown by vapor diffusion at pH 5, by using 3.2 M ammonium sulfate as precipitant. Diffraction data at ultrahigh resolution (0.97 Å) and completeness (99.9%) have been collected under cryogenic conditions, by using synchrotron radiation. The crystals belong to the orthorhombic space group $P2_12_12_1$, with cell constants $a = 37.14$, $b = 39.42$, $c = 44.02$ Å, and one protein monomer per asymmetric unit. Attempts to solve the crystal structure by ab initio methods are in progress. Proteins 28:580–585, 1997** © **1997 Wiley-Liss, Inc.**

Key words: ***B. pasteurii;*** **gram positive; cytochrome c_{553}; purification; electron transfer; crystallization; x-ray diffraction**

INTRODUCTION

The gram-positive, spore-forming, and highly ureolytic soil bacterium *Bacillus pasteurii*[1] is an obligate aerobe and facultative alkaliphile that grows optimally at pH 9.2 in the presence of relatively high amounts of NH_4^+ salts[2,3] or urea.[4,5] Early studies on the respiratory chain of this bacterium showed the presence of membrane-bound cytochromes of the *b* and *c* types in ammonium-grown cells, together with the presence of a terminal oxidase of the aa_3 type.[6,7] In the urea-rich medium, a significant decrease of membrane-bound cytochromes was observed.[6] Recently, a coupling mechanism of the ATP-generating system with urea hydrolysis has been proposed to operate in urea-grown cells, functioning both through alkalinization of the cytoplasm, with consequent activation of ATPase, and also through efflux of ammonium ions, which causes a net increase of the membrane potential, $\Delta\psi$.[8] Overall, little information is available on the proteins involved in the respiratory electron transfer processes in *B. pasteurii*.

During our studies on the structure–function relationships of urease from *B. pasteurii*,[9,10] we discovered significant amounts of a *c*-type cytochrome in the soluble fraction of cells grown in the presence of urea at pH 9 and greater. High levels of membrane-bound cytochromes are known to occur in obligate[11,12] and facultative[13] alkaliphilic bacteria.[14] In the latter, this level rises on increasing the pH of the growth medium,[13,15] indicating an adaptation to environmental stress. The alkaliphilic cytochromes are characterized by distinctly low reduction potentials,[14–16] a feature that has been suggested to facilitate electron transfer to the membrane-bound oxidase in the presence of the large negative membrane potentials associated with the alkaline pH growth medium.[13] Very few cytochromes *c* have been isolated from gram-positive bacteria,[13,17–22] but only in one case is the protein probably strictly soluble,[19] the other cases being actually related to cleaved domains belonging to membrane-anchored proteins. A functional role of these cytochromes in the bacterial respiratory chain has been proposed.[13,14,20] However, in contrast with cytochromes from gram-negative bacteria, for which many details of the structure–function relationships are known,[23] no structural information is available for cytochromes isolated from gram-positive bacteria.

We report here the purification, spectral characterization, crystallization, and preliminary high-resolution x-ray crystallographic data collection and analysis of the soluble *c*-type cytochrome from *B. pasteurii*.

Abbreviations: EDTA, ethylenediamine tetraacetic acid; MWCO, molecular weight cut-off; Tris, tris(hydroxymethyl)-aminomethane; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; FPLC, fast protein liquid chromatography; BCA, bicinchoninic acid; PEG, polyethylenglycol

*Correspondence to: Stefano Ciurli, Institute of Agricultural Chemistry, University of Bologna, Viale Berti Pichat, 10 I-40127 Bologna, Italy.
E-mail: sciurli@agrsci.unibo.it
Received 20 February 1997; Accepted 17 March 1997

© 1997 WILEY-LISS, INC.

MATERIALS AND METHODS

Protein Isolation and Purification

Cells (100 g wet weight) of *B. pasteurii* (DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, Germany, type strain 33) were grown under aerobic conditions, harvested, and lysed as previously described.[9] All the following operations were carried out at 4°C.

Anion exchange chromatography

The soluble extract (ca. 400 ml) was extensively dialyzed against a 50-fold volume of buffer A (50 mM phosphate, pH 7.5, containing 50 mM Na_2SO_3, 1 mM EDTA, and 50 mM NaCl), and then loaded (6 ml/min) onto a Q Sepharose XK 50/20 column (Pharmacia) previously conditioned with buffer A. The column was then eluted with the same buffer until an orange-red unretained fraction, containing a *c*-type cytochrome, was eluted.

Hydrophobic interaction chromatography

The ionic strength of the latter fraction was raised by adding solid $(NH_4)_2SO_4$ up to 2 M and the soluble portion, obtained after centrifugation, was loaded (4 ml/min) onto a Phenyl Sepharose XK 26/20 column (Pharmacia) previously equilibrated with 50 mM phosphate buffer, pH 7.5, containing 2 M $(NH_4)_2SO_4$. After washing the column with five bed volumes of conditioning buffer, a linear gradient (1 L) was developed from 2 M to 0 M $(NH_4)_2SO_4$. The fraction containing the *c*-type cytochrome, eluted at 1.2 M $(NH_4)_2SO_4$, was extensively dialyzed and then concentrated to 2 ml using a pressure ultrafiltration stirred cell (Amicon) equipped with a Spectrum Molecular/Por YM5 membrane (MWCO 5 kDa).

Gel filtration chromatography

The concentrated fraction from the previous step was loaded (1 ml/min) onto a Superdex 75 XK 26/60 column (Pharmacia), preconditioned with 20 mM phosphate buffer, pH 7.5, containing 150 mM NaCl. The fractions containing the *c*-type cytochrome were collected, pooled, dialyzed, and stored at −20°C after solvent exchange with 20 mM Tris · HCl, pH 8.0.

Electrophoresis

SDS polyacrylamide gels (7.5% stacking gel and 15% running gel) were run at 25°C by using the method of Laemmli[24] in a Bio-Rad Mini-PROTEAN II cell. The SDS-PAGE gels were stained by using the Bio-Rad Silver Stain Plus kit. IEF was performed by using a Multiphor Pharmacia horizontal electrophoresis cell at 25°C and precast 7.5% acrylamide gels. The SDS-PAGE and IEF gels were also stained for heme by using tetramethylbenzidine.[25]

The stained gels were probed by using a Bio-Rad GS-670 imaging densitometer, and profile analysis was performed with the Bio-Rad Molecular Analyst 1.01 software. The molecular mass of the cytochrome was estimated with the Bio-Rad broad-range standards. The isoelectric point of the cytochrome was estimated by using the Pharmacia low pI-range standards.

Native Molecular Mass Determination

The native molecular mass of the cytochrome was estimated by using the method of Andrews.[26] The purified *c*-type cytochrome and standard proteins (albumin, ovalbumin, chimotrypsinogen, ribonuclease, and *Rubrivivax gelatinosus* high-potential iron-sulfur protein[27]; 66.2, 45, 13.7, and 7.972 kDa, respectively) were loaded onto an FPLC Superdex 75 HR 10/30 column (Pharmacia) and eluted (0.5 ml/min) by using phosphate buffer, pH 7.5, containing 150 mM NaCl.

Protein Assay

Cytochrome concentration was determined by using the BCA method.[28] Bovine serum albumin was used as standard.

Electronic Spectroscopy

Absorption spectra were recorded with a Jasco 7800 spectrophotometer. The spectrum of the reduced cytochrome was measured in the presence of sodium dithionite. The absorption coefficients (mM^{-1} cm^{-1}) were calculated from the electronic spectra by using a molecular mass of 9.5 kDa.

Protein Crystallization

Crystallization trials on the freshly purified cytochrome were performed at 20°C by the hanging drop method, using 3 µl of a 16-mg/ml protein solution in 20 mM Tris · HCl, pH = 8.0, and diluting this volume with 3 µl of precipitant solution. The drop was equilibrated by vapor diffusion against 1 ml of precipitant solution by using an Hampton Research 24-well Linbro plate.

Crystallographic Diffraction Data Collection and Evaluation

A single crystal of cytochrome c-553, with dimensions of 0.2 × 0.2 × 0.6 mm, was transferred from the mother liquor to the cryobuffer (20% glycerol in the precipitant solution). After about 1 minute the crystal was scooped up in a rayon cryoloop and rapidly exposed to a cold nitrogen stream (Oxford Cryosystem) on the BW7B wiggler line (λ = 0.8855 Å) of the DORIS storage ring at the EMBL outstation at the Deutsches Elektronen Synchrotron (DESY) in Hamburg (Germany). Diffraction data were collected at 100 K by using a 30-cm MAR Research imaging plate scanner (Hamburg, Germany). The crystal was mounted with a* approximately along the spindle axis. One crystal was used to record the entire data set in three sweeps, at different exposure times, to accurately record both the strongest low-resolution

TABLE I. Purification of *B. pasteurii* Cytochrome c-553

Step	Protein (mg)	A_{280}/A_{411}
Crude extract	3640	29
Q Sepharose	86	3.9
Phenyl Sepharose	15	0.27
Superdex 75	12	0.19

and the weakest high-resolution diffraction intensities. The images were processed with DENZO[29] and merged with SCALEPACK.[30]

RESULTS AND DISCUSSION

Protein Purification and Characterization

The purification to homogeneity of the *c*-type cytochrome from *B. pasteurii* was carried out by using a sequence of standard chromatographic techniques. Fractions containing the cytochrome, eluted from the first anion-exchange chromatography, were loaded subsequently onto an hydrophobic interaction column and a size-exclusion chromatography column. All steps significantly improved the purity (Table I). The purified cytochrome migrated as a single band during SDS-PAGE and IEF experiments, indicating that the protein thus obtained was homogeneous. The yield of pure protein was about 12 mg per 100 g of wet cell paste. The molecular mass was 9.5 kDa when estimated by SDS-PAGE and 19.5 kDa when measured in nondenaturating conditions by using size-exclusion techniques, suggesting an apparent dimerization in solution. IEF experiments allowed the observation of a single heme-staining band with a pI = 3.3.

The electronic absorption spectra are shown in Figure 1A. The spectrum of the isolated protein is typical of an oxidized low-spin *c*-type cytochrome containing an hexa-coordinated Fe^{3+} ion[23] with maxima at 552 (ϵ = 6.7), 524 (ϵ = 9.8), 411 (ϵ = 114.6), 357 (ϵ = 28.7), and 280 nm (ϵ = 20.2). No changes in the spectrum occurred after addition of hexacyanoferrate(III), whereas addition of sodium dithionite induced the appearance of a characteristic spectrum of a reduced low-spin cytochrome c, with a sharp α band at 553 nm (ϵ = 20.5) and additional maxima at 523 (ϵ = 15.1) and 416 (ϵ = 151) nm. We denote the protein as cytochrome c-553, until a classification based on amino acid sequence, presently in progress, is available. The above observations suggest that cytochrome c-553 has a low reduction potential. The reduced-minus-oxidized spectrum is reported in Figure 1B, showing maxima at 553, 523, and 418 nm, along with a pronounced minimum at 405 nm. This difference spectrum shows isosbestic points at 560, 542.5, 532.5, 509, 438, and 411.5 nm.

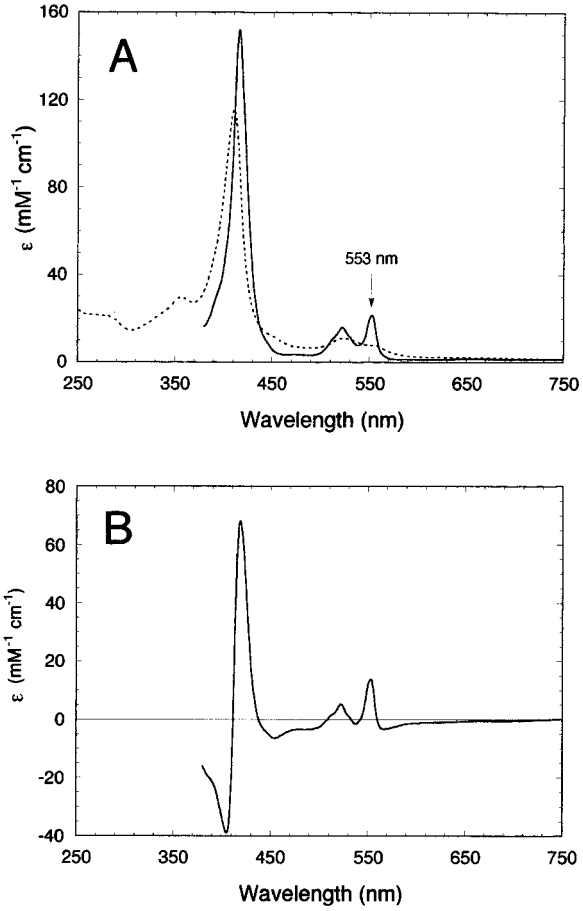

Fig. 1. **A:** Electronic absorption spectrum of reduced (continuous line) and oxidized (dotted line) of *B. pasteurii* cytochrome c-553. **B:** The reduced minus oxidized difference spectrum is also reported.

Protein Crystallization and Diffraction Data Evaluation

The initial screening for determination of the crystallization conditions was carried out using 100 mM sodium acetate (pH 4 and 5), 100 mM sodium citrate (pH 6.3 and 7), or 100 mM Tris · HCl (pH 8 and 9) buffers containing either 1.6–2.9 M $(NH_4)_2SO_4$ or 50–100% (v/v) of a 30% (w/v) PEG 6000 (Sigma) stock solution as precipitant. No formation of precipitate was observed in these conditions, even when 1 M LiCl was used together with PEG as coprecipitant. Variable amounts of amorphous material precipitated both above and below pH 5, using the same buffer systems containing 3.2 M ammonium sulfate as precipitant. However, successful and reproducible crystallizations were obtained using 100 mM sodium acetate at pH 5 containing 3.2 M ammonium sulfate. Under these conditions, regularly shaped rods grew to about 0.2 × 0.2 × 0.6 mm on average after 1 week.

Although crystals of cytochrome c-553 diffract well in the synchrotron beam at room temperature with-

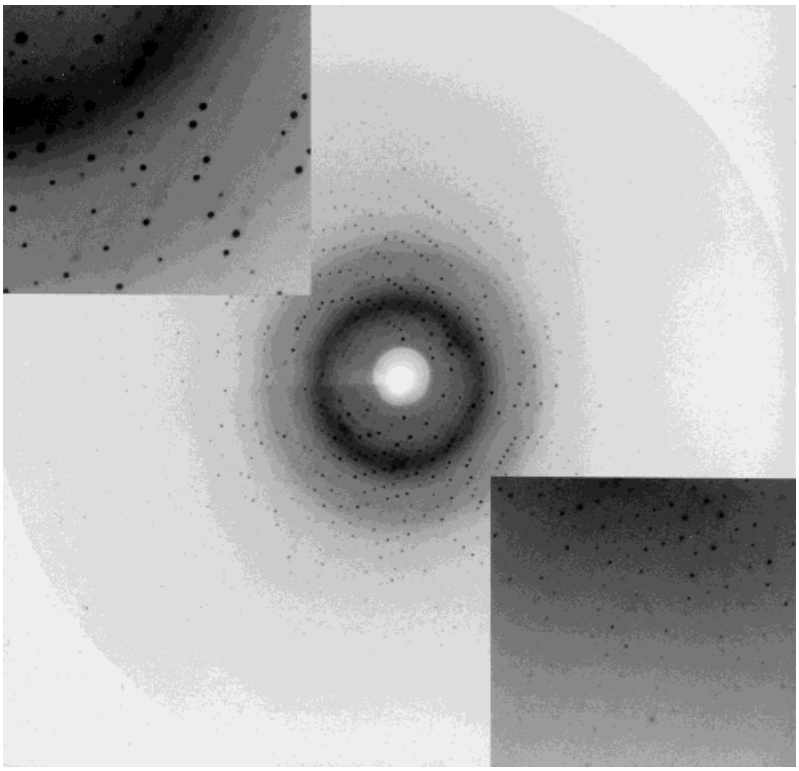

Fig. 2. Diffraction pattern from an oscillation image of cytochrome c-553 from *B. pasteurii.* Insets show details of the image at higher magnification.

out signs of radiation damage, data were collected under cryogenic conditions to reduce thermal motion and to increase the quality and resolution of the data.[31–33] Very high resolution (0.97 Å) and completeness (99.9%) were thus obtained. The quality of the diffraction obtained is evident in the image shown in Figure 2 and in the data summary given in Table II. Overall, only 53 reflections are missing, most of them between 1.4 and 1.2 A.

B. pasteurii cytochrome c-553 crystallizes in the orthorhombic space group $P2_12_12_1$, with unit cell dimensions a = 37.14, b = 39.42, c = 44.02 Å. Assuming one molecule (9.5 kDa) per asymmetric unit, the volume-to-mass ratio, V_M, is 1.7 $Å^3$/Da, giving a solvent content of 28%. These values are in the low range found for proteins[34] and indicate a high density of crystal packing and solvent exclusion.

Although the wavelength was optimized for collection of atomic resolution data and no effort was made to optimize the anomalous signal, the Patterson map calculated with coefficients $[F(+) - F(-)]^2$ clearly showed a peak at 8 σ level (Fig. 3), corresponding to the anomalous signal from the single Fe ion. Given the high quality of the data, the atomic resolution obtained, and the presence of anomalous scatterers in the protein, we are proceeding to solve the structure by ab initio methods.

TABLE II. Summary of X-Ray Data Collection on *B. pasteurii* Cytochrome c-553

Space group	$P2_12_12_1$
Unit cell parameters:	
a (Å)	37.14
b (Å)	39.42
c (Å)	44.02
Beam line at DORIS	BW7B
Temperature (K)	100
Lowest resolution (Å)	20
Maximum resolution (Å)	0.97
Wavelength (Å)	0.8855
Number of images	179
Oscillation range (°)	0.8–3
R_{merge}	0.074
Raw measurements used	212,694
Unique reflections	38,959
% Completeness	99.9
% Completeness in highest resolution bin (0.99–0.97)	99.6
% Greater than 3 σ	86.3
I/σ in highest resolution bin (0.99–0.97)	4.18

This result will represent the first structural characterization of a soluble cytochrome isolated from gram-positive bacteria and will provide, at atomic resolution, the basis for understanding the peculiar features of this class of proteins.

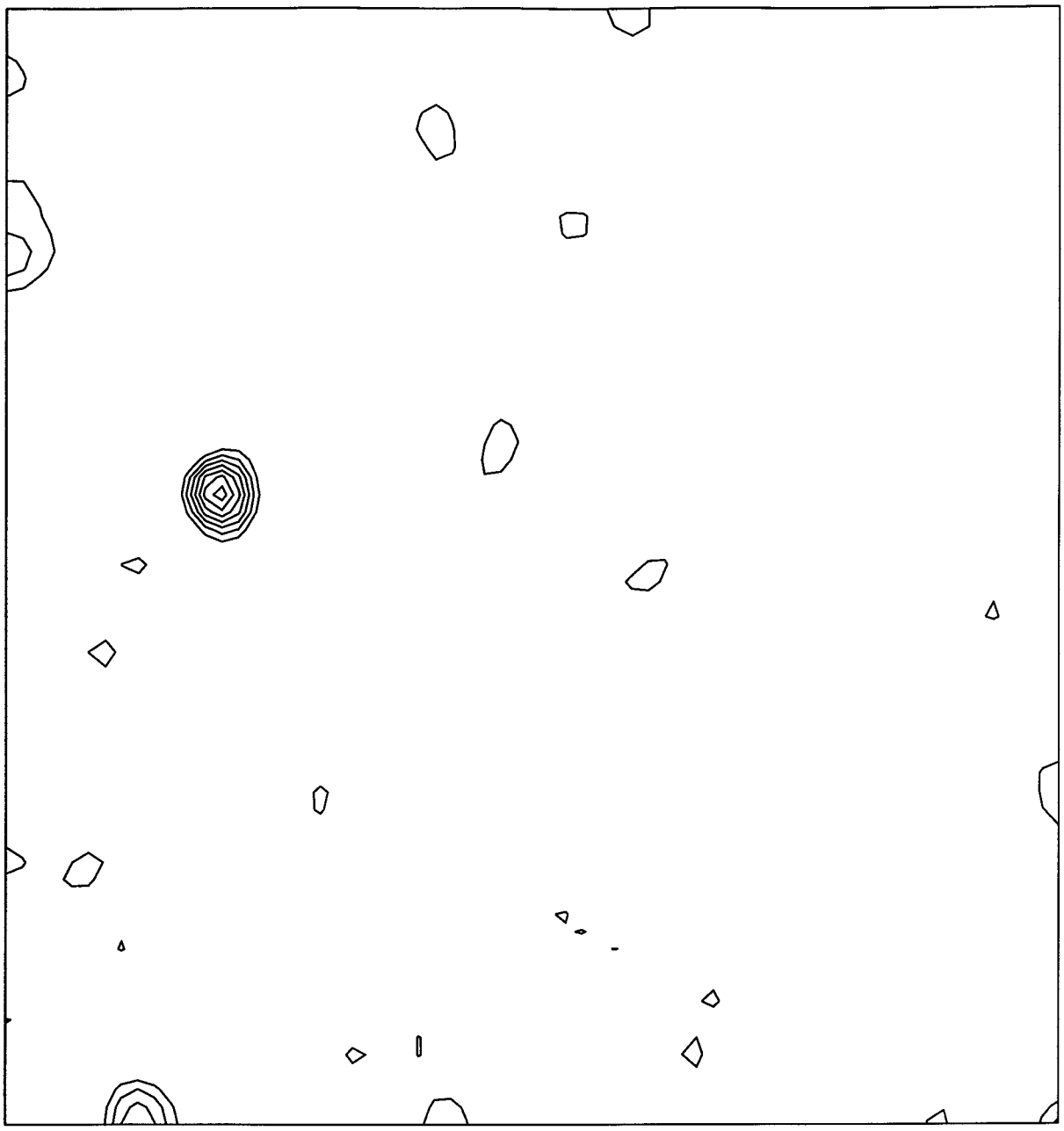

Fig. 3. $\omega = \frac{1}{2}$ Harker section of the anomalous Patterson map calculated using all data between 0.97 and 20 Å. The map is contoured at 1 σ intervals, starting at 2 σ.

ACKNOWLEDGMENTS

The authors thank the European Union for support of the work at EMBL Hamburg through the HCMP Access to Large Installation Project, Contract CHGE-CT93-0040. S.C. is grateful to Consiglio Nazionale delle Ricerche, Comitato 11 "Biotecnologie e Innovazione," for partially funding this research. The authors thank Dr. A. Hochkoeppler for performing the isoelectric focusing experiment, Prof. S. Mangani for helpful discussions about protein crystallization, and Prof. D. Zannoni for critically reading the manuscript.

REFERENCES

1. Gibson, T. An investigation of the *Bacillus pasteurii* group. III. Systematic relationship of the group. J. Bacteriol. 29:491–502, 1935.
2. Wiley, W.R., Stokes, J.L. Requirement of an alkaline pH and ammonia for substrate oxidation by *Bacillus pasteurii*. J. Bacteriol. 84:730–734, 1962.
3. Wiley, W.R., Stokes, J.L. Effect of pH and ammonium ions

on the permeability of *Bacillus pasteurii.* J. Bacteriol. 86:1152–1156, 1963.
4. Larson, A.D., Kallio, R.E. Purification and properties of bacterial urease. J. Bacteriol. 68:67–73, 1954.
5. Bornside, G.H., Kallio, R.E. Urea-hydrolyzing bacilli. I. A physiological approach to identification. J. Bacteriol. 71: 627–634, 1956.
6. Haddock, B.A., Cobley, J.G. Electron transport in the alkalophile *Bacillus pasteurii* (N.C.I.B. 8841). Biochem. Soc. Trans. 4:709–711, 1976.
7. Hoddinott, M.H., Reid, G.A., Ingledew, W.J. The respiratory chain and proton electrochemical gradient in the alkalophile *Bacillus pasteurii.* Biochem. Soc. Trans. 6:1295–1298, 1978.
8. Jahns, T. Ammonium/urea-dependent generation of a proton electrochemical potential and synthesis of ATP in *Bacillus pasteurii.* J. Bacteriol. 178:403–409, 1996.
9. Benini, S., Gessa, C., Ciurli, S. *Bacillus pasteurii* urease: An heteropolymeric enzyme with a binuclear nickel active site. Soil Biol. Biochem. 28:819–821, 1996.
10. Benini, S., Ciurli, S., Nolting, H.F., Mangani, S. X-ray absorption spectroscopy study of native and phenylphosphorodiamidate-inhibited *Bacillus pasteurii* urease. Eur. J. Biochem. 239:61–66, 1996.
11. Guffanti, A.A., Susman, P., Blanco, R., Krulwich, T.A. The protonmotive force and α-aminoisobutyric acid transport in an obligately alkalophilic bacterium. J. Biol. Chem. 253: 708–715, 1978.
12. Lewis, R.J., Belkina, S., Krulwich, T.A. Alkalophiles have much higher cytochrome contents than conventional bacteria and than their own non-alkalophilic mutant derivatives. Biochem. Biophys. Res. Commun. 95:857–863, 1980.
13. Yumoto, I., Fukumori, Y., Yamanaka, T. Purification and characterization of two membrane-bound *c*-type cytochromes from a facultative alkalophilic *Bacillus.* J. Biochem. 110:267–273, 1991.
14. Hicks, D.B., Krulwich, T.A. The respiratory chain of alkaliphilic bacteria. Biochim. Biophys. Acta 1229:303–314, 1995.
15. Lewis, R.J., Prince, R.C., Dutton, P.L., Knaff, D.B., Krulwich, T.A. The respiratory chain of *Bacillus alcalophilus* and its nonalkalophilic mutant derivative. J. Biol. Chem. 256:10543–10549, 1981.
16. Kitada, M., Lewis, R.J., Krulwich, T.A. Respiratory chain of the alkalophilic bacterium *Bacillus firmus* RAB and its nonalkalophilic mutant derivatives. J. Bacteriol. 154:330–335, 1983.
17. Jacobs, A.J., Kalra, V.K., Cavari, B., Brodie, A.F. Purification, physicochemical properties, and localization of cytochrome c in energy-transducing membranes from *Mycobacterium phlei.* Arch. Biochem. Biophys. 194:531–541, 1979.
18. Woolley, K.J. The *c*-type cytochromes of the gram-positive bacterium *Bacillus licheniformis.* Arch. Biochem. Biophys. 254:376–379, 1987.
19. Davidson, M.W., Gray, K.A., Knaff, D.B., Krulwich, T.A. Purification and characterization of two soluble cytochromes from the alkalophile *Bacillus firmus* RAB. Biochim. Biophys. Acta 933:470–477, 1988.
20. Sone, N., Kutoh, E., Yanagita, Y. Cytochrome c-551 from the thermophilic bacterium PS3 grown under air-limited conditions. Biochim. Biophys. Acta 977:329–334, 1989.
21. von Wachenfeldt, C., Hederstedt, L. *Bacillus subtilis* 13-kilodalton cytochrome c-550 encoded by *ccc* A consist of a membrane-anchor and heme domain. J. Biol. Chem. 265: 13939–13948, 1990.
22. Hreggvidsson, G.O. Two structurally different cytochromes c from *Bacillus azotoformans:* On the evolution of gram-positive bacteria. Biochim. Biophys. Acta 1058:52–55, 1991.
23. Moore, G.R., Pettigrew, G.W. "Cytochrome c: Evolutionary, Structural and Physicochemical Aspects." Berlin: Springer-Verlag, 1990.
24. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970.
25. Thomas, P.E., Ryan, D., Levin, W. An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. Anal. Biochem. 75:168–176, 1976.
26. Andrews, P. Estimation of molecular weights of proteins by sephadex gel-filtration. Biochem. J. 91:222–233, 1964.
27. Tedro, S.M., Meyer, T.E., Kamen, M.D. Primary structure of a high potential iron-sulfur protein from the purple non-sulfur photosynthetic bacterium *Rhodopseudomonas gelatinosa.* J. Biol. Chem. 251:129–136, 1976.
28. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Garther, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76–85, 1985.
29. Gewirth, D., Otwinowski, Z., Minor, W. "The HKL Manual." Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, 1995.
30. Rees, D.C. Intensity statistics. Acta Cryst. Sect. A. 50:157–163, 1980.
31. Hope, H. Cryocrystallography of biological macromolecules at ultra-low temperature. Annu. Rev. Biophys. Biophys. Chem. 19:107–126, 1990.
32. Watenpaugh, K.D. Macromolecular crystallography at cryogenic temperatures. Curr. Opin. Struct. Biol. 1:1012–1015, 1991.
33. Rogers, D.W. Cryocrystallography. Structure 2:1135–1139, 1994.
34. Matthews, B.W. Solvent content of protein crystals. J. Mol. Biol. 33:491–497, 1968.