Pseudomonas aeruginosa aliphatic amidase is related to the nitrilase/cyanide hydratase enzyme family and Cys^{166} is predicted to be the active site nucleophile of the catalytic mechanism

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Abstract **A database search indicated homology between some members of the nitrilase/cyanide hydratase family, *Pseudomonas aeruginosa* and *Rhodococcus erythropolis* amidases and several other proteins, some of unknown function. BLOCK and PROFILE searches confirmed these relationships and showed that four regions of the *P. aeruginosa* amidase had significant homology with corresponding regions of nitrilases. A phylogenetic tree placed the *P. aeruginosa* and *R. erythropolis* amidases in a group with nitrilases but separated other amidases into three groups. The active site cysteine in nitrilases is conserved in the *P. aeruginosa* amidase indicating that Cys^{166} is the active site nucleophile.**

Key words: Amidase; Nitrilase; Phylogenetic tree; Active site prediction; *Pseudomonas aeruginosa*; *Rhodococcus erythropolis*

1. Introduction

The inducible aliphatic amidase (acylamide amidohydrolase; EC 3.5.1.4) of *Pseudomonas aeruginosa* catalyses the hydrolysis of aliphatic amides with short acyl chains to produce the corresponding acids and ammonium [1]. The amino acid sequence [2] has 80% identity and 90% similarity with *Brevibacterium* sp R312 wide-spectrum amidase [3] and these two belong to a group which also probably includes amidases from *Methylophilus methylotrophus* [4], *Alcaligenes xylogenes* and *Pseudomonas cepacia* (Novo, C., unpublished). The *P. aeruginosa* enzyme is notable for the variations in its substrate specificity conferred by point mutations [5].

Many other amidases unrelated to the *P. aeruginosa* group have been sequenced. Ten [6–9] contain a highly conserved central region rich in glycine, serine and alanine residues which give the consensus pattern, known in the PROSITE dictionary as amidase signature (PDOCOO494;PS00571).

The catalytic mechanism for the *P. aeruginosa* amidase is not known but the participation of a thiol group in the catalytic mechanism was proposed for the amidase from *Brevibacterium* sp R312 [10] based on the work of Jallageas et al. [11]; however, no particular cysteine residue was assigned as the active site nucleophile and no conclusive evidence was given for the proposed mechanism.

In some organisms conversion of aliphatic nitriles (organic compounds containing a CN moiety) to acid and ammonium is a two-step process. The first, catalysed by a nitrile hydratase, produces an amide whose hydrolysis is then catalysed by an amidase. Nitrile hydratases are not structurally related to amidases or to nitrilases [7,12,13]. Nitrilases catalyse the direct cleavage of nitriles to the corresponding acids and ammonium. Several microbial and plant nitrilases that use aromatic nitriles as substrates are known [14–20] and one from *R. rhodochrous* uses aliphatic nitriles as substrates [21]. The amino acid sequences of several nitrilases are known [14–21]; they are susceptible to inactivation by thiol reagents and the Cys residue acting as the active site nucleophile has been identified [15,21]. Two fungal cyanide hydratases from *Gloeoceocospora sorghi* [22] and *Fusarum lateritum* [23] have extensive amino acid sequence homology with nitrilases and two conserved regions, known as nitrilase/cyanide hydratase signatures (PDOCOO712; PS00920/1) in the PROSITE dictionary, are found in all members of the family.

Until this report no significant homologies had been reported between the *P. aeruginosa* amidase group and any other enzymes.

2. Materials and methods

2.1. Amino acid sequence homology

Searches for homology in databases were done with PROSRCH version 1.1, at the Biocomputing Research Unit in Molecular Biology, University of Edinburgh, BLAST [24], at the National Center for Biotechnology Information (NCBI), Bethesda, USA, and FASTA [25], at the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany. The data banks available at the above institutions were used in conjunction with the programmes.

2.2. Blocks/blockmaker programs

The search on the BLOCKS database version 8.0 derived from PROSITE 12 keyed to SWISS-PROT 29 [26,27] and was done by the BLOCKS search program version 1.5 [28]. The search of blocks common to aligned sequences was done using BLOCKMAKER program version 1.11 [29] which uses the Smith's MOTIF program [29] and a modification of Lawrence's Gibbs sample program [30]. Both programs are from the Fred Hutchinson Cancer Research Center, Seattle, USA.

2.3. Profile analysis

The nitrilase profile was constructed by applying successively the programs LINEUP, PILEUP and PROFILEMAKE version 4.40 to six nitrilase and two cyanide hydratase sequences in databases. Nitrilases: *Arabidopsis thaliana* [17,19]; *Alcaligenes faecalis* [14]; *Rhodococcus rhodochrous* [15,21]; *Klebsiella pneumoniae* [18]. Cyanide hydratases:

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Abbreviations: PDOC, Prosite documentation; PS, PROSITE; PAM probability matrix, corresponds to one accepted amino acid substitution per hundred sites.

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G. sorghi [22]; *F. laterittum* [23]. The specificity of the profile was checked in PIR and SWISS-PROT databases using the program PROFILESEARCH. The comparison of protein sequences with the nitrilase profile was done with PROFILEGAP and with the profile library using PROFILESCAN.

All the programs used are included in GCG package version 7.3 [31].

2.4. Phylogenetic relationships

The phylogenetic tree of protein sequences was constructed by Phylotree and Rootedtree programs at the Darwin Computational Biochemistry Research Group (CBRG) ETH, Zurich, Switzerland.

2.5. Protein structure predictions

Hydrophilicity was determined by the method of Kyte and Doolittle [32] and solvent accessibility by the method of Rost and Sander [33,34].

2.6. Enzymes/proteins

Enzymes mentioned in the main text are represented by SWISS-PROT database names in Figs. 1 and 3 as follows: Nitrilases: *R. rhodochrous* NRL1-RHORH [15], NRL2-RHORH [21]; *A. thaliana* NRL1-ARATH [17], NRL2-ARATH [19]; *A. faecalis* NRLA-ALCFA [14]; *K. pneumoniae* NRLB-KLEPN [18]. Cyanide hydratases: *G. sorghi* CYHY-GLOSO [22]; *F. laterittum*: CYHY-FUSLA [23]. Amidases: *P. aeruginosa* ALAM-PSEAE [2]; *Rhodococcus erythropolis*; ALAM-RHOER [3,7]. *Saccharomyces cerevisiae* hypothetical protein 1 HYP.PROT.1 (Doignon, F. et. al., unpublished). *Staphylococcus aureus* hypothetical protein 5 HYP.PROT.5 (Kornblum et al., unpublished). *Staphyloccus lugdunensis* open reading frame 5′-ORF5′ [35]. *Rattus norvegians* β-ureidopropionase BUP-RAT [36].

3. Results and discussion

3.1. Database search

Around 120 sequences, the output of a data base search for proteins showing greatest homology with *P. aeruginosa* amidase, were analysed further by the method of Sander and Schneider [37] to investigate whether any of these homologies implied a structural relationship. Ten sequences lay above the homology threshold, *t*, defined in this method indicating possible structural homology with amidase. One of these sequences was the amidase from *R. erythropolis* which has an identical sequence to the amidase from *Brevibacterium* sp strain R312 [3,7]. Two nitrilases from *R. rhodochrous* [15,21] appeared above the threshold line. Other sequences with implied homology to the *Pseudomonas* amidase emerging from this study included an unascribed gene from *S. lugdunensis* [35] with an identity of 32% in a length of 93 residues and hypothetical protein 5, from *S. aureus* (Kornblum, R. et al., unpublished) with an identity of 32% in a length of 91 residues.

These results provoked a further investigation by doing a BLOCKS search in the *P. aeruginosa* amidase sequence. This detected four blocks (BL00920: A, B, C, D) of the nitrilase/cyanide hydratase family. The blocks aligned with the amidase sequence in the order they were in nitrilases and with similar intervening distances. The probabilities supplied by the programme showed that chance alignment for these blocks was highly unlikely. By combining the results from the BLOCKS search with database homology searches four regions common to nitrilases and the amidases from *P. aeruginosa* and *R. erythropolis* were defined (Fig. 1).

The high homology of the hypothetical protein 1 from *S. cerevisiae* (Doignon, F. et al., unpublished) with nitrilase sequences, noted previously for the *A. thaliana* nitrilases [20], was confirmed by detection of three blocks BL00920; E, B, C from the nitrilase family in the correct order in its sequence (data not shown).

3.2. Profile analysis

In order to carry out a profile analysis of the *P. aeruginosa* amidase sequence, a nitrilase profile was constructed and its specificity checked by using it to search the PIR and SWISS-PROT databases. The nitrilase profile showed a high specificity for the nitrilase family: 8 sequences were detected in the SWISS-PROT databank ($40.69 < z$ score[1] < 49.72) and 7 sequences were detected in the PIR databank, ($42.43 < z$ score < 48.10) (data not shown). The profile search also detected the *P. aeruginosa* amidase with the best *z* score (6.04) after nitrilases in the SWISS-PROT databank and with the fifth best in the PIR databank (*z* score = 5.89). The hypothetical protein 5 sequence from *S. aureus* had the second best *z* score (8.47) after nitrilases in the PIR databank. The profile search also detected other sequences presumably related to the nitrilase family: the *R. norvegians* β-ureidopropionase [36] with second best *z* score (5.44) after nitrilases in the SWISS-PROT databank and hypothetical protein 1 from *S. cerevisiae* with the best *z* score (23.10) after nitrilases in the PIR databank.

3.3. Phylogenetic tree

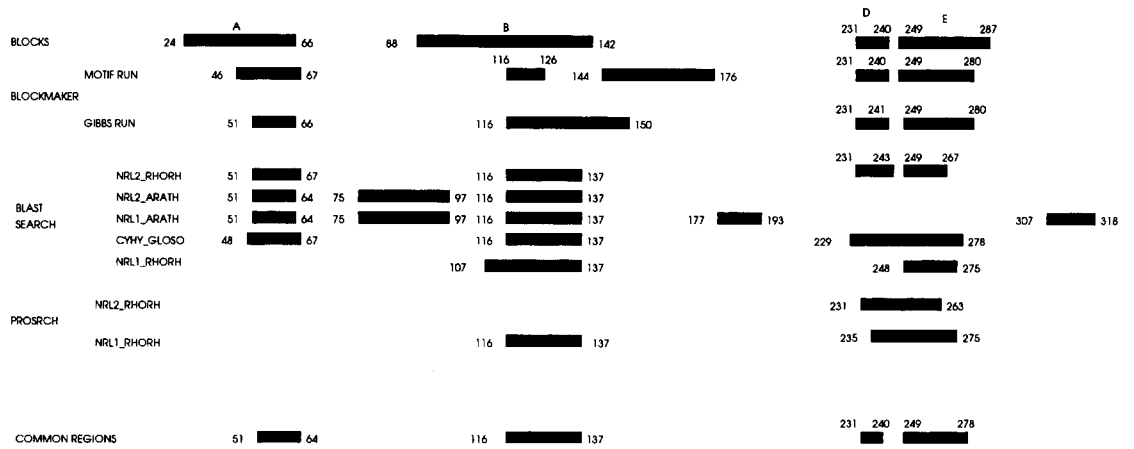
Using Phylotree and Rooted tree programs, a phylogenetic tree (Fig. 2) was constructed of amidases, nitrilases, nitrile hydratases, acyl transferases (amidases have acyl transferase activity), ureases and the other proteins found to share homology with nitrilases. Amidases were separated into four categories. The signature group together with the amidase from *Aspergillus oryzae* [38] comprised amidases exclusively. Amidases from *P. aeruginosa* and *R. erythropolis* were included with the nitrilase family; an amidase from *Mycobacterium smegmatis* [39] was related to ureases and another, the putative amidase from *Salmonella typhimurium* (Xu, K., unpublished), was separated from the others. Nitrilases and nitrile hydratases were unrelated, in agreement with the conclusions of other authors [21].

Inspection of the proteins shown by the tree to be related to the nitrilase family suggested that: (i) β-ureidopropionase diverged first (114 PAM distant) from an ancestral gene; (ii) the *S. aureus* and *S. lugdunensis* sequences were phylogenetically very close and shared a common ancestor with the acyltransferase sequence from *E. coli* [40,41]; (iii) the hypothetical protein 1 from *S. cerevisiae* was closely related to the two nitrilases from *A. thaliana*; (iv) the amidases from *P. aeruginosa* and *R. erythropolis* were, out of all the sequences of the group, the most closely related to the nitrilase family.

3.4. P. aeruginosa *amidase active site*

The active site of nitrilases contains a cysteine –SH that is proposed to carry out a nucleophilic attack on the nitrile C atom during the catalytic cycle [42,43]. Comparisons between the nitrilase profile sequence and those of each of the other sequences assigned to the nitrilase phylogenetic group showed that, with the exception of the *E. coli* acyltransferase, all the others had a cysteine residue matching the nitrilase cysteine active site nucleophile (data not shown). The same approach applied to each of the amidases in the amidase signature group, and those from *A. oryzae* and *S. typhimurium*, revealed that

[1]All sequence alignments had a *z* score > 2.50 (maximum *z* score = 49.2), where *z* represents the difference in standard deviation units between the comparison score and the score for sequences unrelated to the profile.



ALAM_PSEAE	51	**GMDLVVFPE**YSLQ**G**	64		116	**Y**NTLVL**I**DNN**G**EIVQKY**RKI**IP	137
ALAM_RHOER	51	**GMDLVVFPE**YSTQ**G**	64		116	**Y**NTLIL**I**DNK**G**EIVQRY**RKI**LP	137
CYHI_FUSLA	38	**GCKFVAFPE**VWIP**G**	51		208	**I**QPLTW**L**LLS**M**LSRLAR**GLL**LP	229
CYHY_GLOSO	38	**GCKLIAFPE**VWIP**G**	51		110	**Y**LTQVL**I**SPL**G**DVINHR**RKI**KP	131
NRL1_ARATH	57	**GAELVLFPE**GFIG**G**	70		134	**Y**CTVLF**F**SPQ**G**QFLGKH**RKL**MP	155
NRL1_RHORH	45	**GAEFLAFPE**VWIP**G**	58		118	**Y**LSQVF**I**DQN**G**DIVANR**RKL**KP	139
NRL2_ARATH	50	**GSELVVFPE**AFIG**G**	63		127	**Y**CTALF**F**SPQ**G**QFLGKH**RKL**MP	148
NRL2_RHORH	39	**GCELVAFPE**VFIP**G**	52		112	**Y**MTQLV**I**DAD**G**QLVARR**RKL**KP	133
NRLA_ALCFA	39	**GCDLIVFGE**TWLP**G**	52		111	**Y**LGQCL**I**DDK**G**QMLWSR**RKL**KP	132
NRLB_KLEPN	37	**GAQLVAFPE**LWIP**G**	50		109	**Y**MSQML**I**DAD**G**ITKIRR**RKL**KP	130

ALAM_PSEAE	231	**GHSAI**IGF**DG**	240	249	**EEMGI**QY**A**Q**L**S**L**SQIRDA**R**ANDQSQN**H**LF**K**	278
ALAM_RHOER	231	**GHSAI**IGF**DG**	240	249	**EEYGI**QY**A**Q**L**S**V**SAIRDA**R**ENDQSQN**H**IF**K**	270
CYHY_FUSLA	257	**GHARI**YRP**DG**	266	275	**DFDGLLFVDIDLNECHLTKVLADFAG**H**YM**R**	296
CYHY_GLOSO	256	**GHARI**FRP**DG**	265	274	**DFDGLMYVDIDLNESHLTKALADFAG**H**YM**R**	295
NRL1_ARATH	268	**GGSVI**ISP**LG**	277	286	**ESEGLVTADIDLGDIARAKLYFDSVGYYS**R**	307
NRL1_RHORH	258	**GWARI**YGP**DG**	267	277	**DAEGLLYAELDLEQIILAKAAADPAG**H**YS**R**	298
NRL2_ARATH	261	**GGSVI**ISP**LG**	270	279	**ESEGLITADLDLGDVARAKLYFDSVG**H**YS**R**	300
NRL2_RHORH	251	**GFARI**IGP**DG**	260	270	**DEEGILYADIDLSAITLAKQAADPVG**H**YS**R**	291
NRLA_ALCFA	250	**GSSMI**FAP**DG**	259	269	**DAEGLIIADLNMEEIAFAKAINDPVG**H**YS**K**	290
NRLB_KLEPN	244	**GYARI**YGP**DM**	253	263	**TEEGIVYAEIDLSMLEAAKYSLDPTG**H**YS**R**	284

Fig. 1. Regions of amino acid homology common to the nitrilase/cyanide hydratase family and the aliphatic amidases from *P. aeruginosa* and *R. erythropolis*. The upper part of the figure shows a compilation of the BLOCK, BLOCKMAKER and database searches for sequence homology; the *P. aeruginosa* amidase numbering is used. Below, the sequences of the four common regions are displayed with residues that are similar or identical in most or all sequences shown in bold. See section 2 for identification of the enzymes.

only *Pseudomonas syringae* indoleacetamide hydrolase [44] had a cysteine matching with the nitrilase active site cysteine. However, this enzyme displayed low homology with the nitrilase profile suggesting that the cysteine residue may not have a role as active site nucleophile.

Multiple alignment of group sequences (Fig. 3), confirmed the conservation of the cysteine residue and of a proline, six residues away. Nitrilases showed strong conservation of the sequence C(WA)E whereas for the other members of the group C(D,Y)(DG) was strongly conserved.

Hydrophobicity plots (data not shown) indicated that in all sequences the cysteine residue was in a region with a solvent relative accessibility less than 9% suggesting that the Cys residue was buried in a hydrophobic pocket.

4. Conclusions

The data strongly suggested that amidases from *P. aeruginosa* and *R. erythropolis* (*Brevibacterium* sp. R312 wide-spectrum amidase) were closely related to the nitrilase/cyanide hydratase family. Furthermore the relationship indicated that both amidases use Cys^{166} as the active site nucleophile. By site-directed mutagenesis, we have obtained amidases from *P. aeruginosa* with the changes C166S and C166A. In both cases the mutated enzyme is without activity and is immunologically indistinguishable from the wild-type enzyme (Brown, P. et al., in preparation) strongly supporting the conclusions of this computer-based study. Kobayashi et al. [21] noted that the aliphatic nitrilase of *R. rhodochrous* K22 exhibited relatively

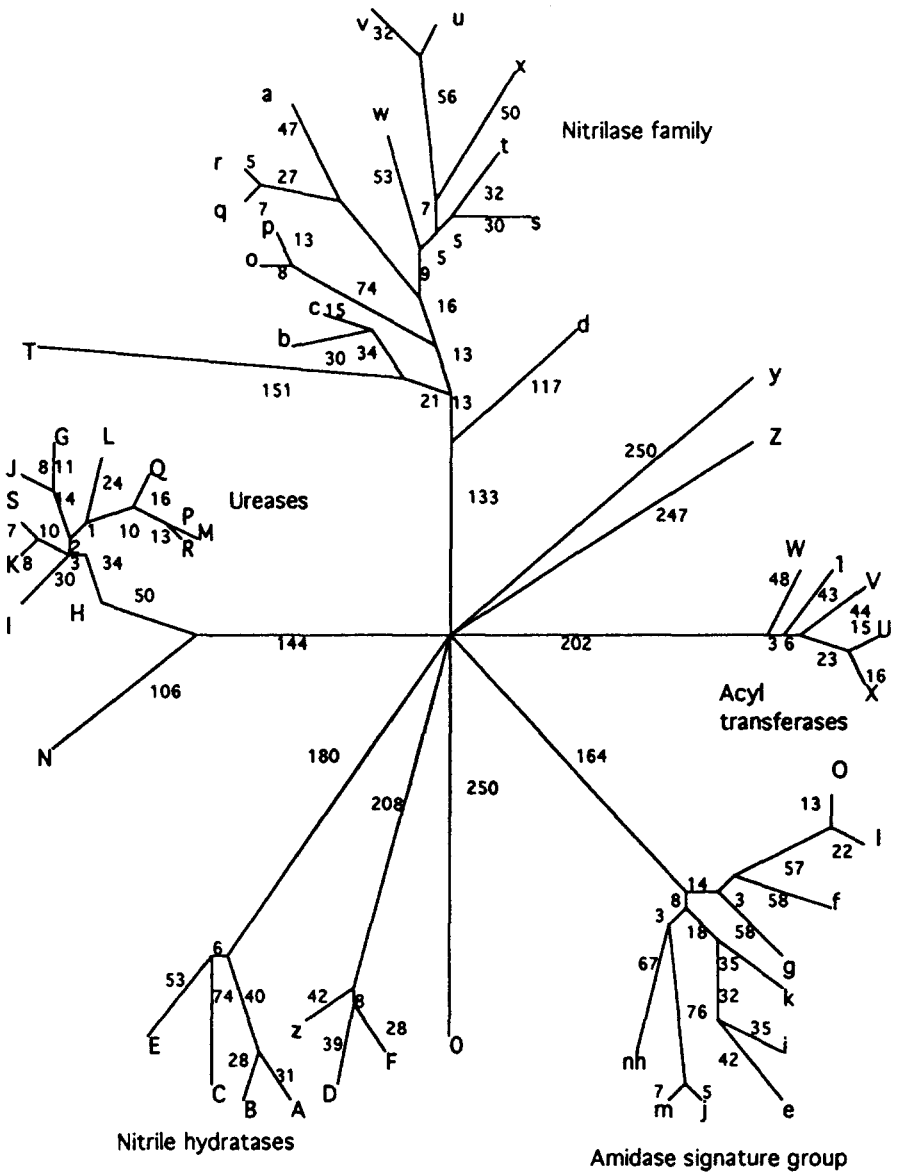


Fig. 2. Phylogenetic tree. Sequences are clustered by the program into groups of related proteins. In the figure, groups are identified by the type of enzyme predominating within the group. Numbers represent PAM values. Sequences not mentioned in the main text or in section 2 are referred to solely by their names in the SWISS-PROT database. *Amidase signature group*: (e) AMID-PSECL, (f) AMID-YEAST, (g) HYIN-BRAJA, (h) NYLA-PSES8, (i) AMID-RHOER, (j) HYIN-AGRT3, (k) *P. syringae* indoleacetamide hydrolase [44], (l) AMDS-EMENI, (m) HYN-AGRT4, (n) NYLA-FLASP, (o) *A. oryzae* amidase [38]; *Nitrilase family*: Nitrilases (q) *A. thaliana* [17], (r) *A. thaliana* [19], (s) *R. rhodococcus* [15], (t) *R. rhodococcus* [21]; Cyanide hydratases (u) *G. sorghi* [22], (v) *F. laterittum* [23]; Nitrilases: (w) *A. faecalis* [14], (x) *K. pneumoniae* [18], (o) *P. aeruginosa* amidase [2], (p) *R. erythropolis* amidase [3,7], (a) *S. cerevisiae* hypothetical protein 1, (b) *S. aureus* hypothetical protein 5, (c) *S. lugdunensis* Orf5′ [35], (d) *R. norvegians* β-ureidopropionase [36]; *Ureases*: (G) UREA-CANEN, (H) URE1-LACFE, (I) URE1-UREUR, (J) UREA-SOYBN, (K) URE1- YEREN, (L) URE2-HELPY, (M) URE1-PROVU, (P) URE1-ECOLI, (Q) URE1-KLEAE, (R) URE1-PROMI, (S) URE1-MORMO, (N) *M. smegmatis* amidase [39]; *acetyl and acyl transferases*: (U) CAT-CLOBU, (V) CAT1-STAAU, (W) CAT2-HAEIN, (X) CAT1-CLOPE, (Y) ATDA- MESAU, (Z) ATDA-HUMAN, (0) IIK-SOLTU, (1) CAT-BACPU; *nitrile hydratases*: (A) NHAB-PSECL, (B) NHAB-RHOER, (C) NHB1- RHORH, (D) NHA1-RHORH, (E) NHB2-RHOERH, (F) NHA2-RHORH. *Enzymes separate from main groupings*: (y) *S. typhimurium* amidase (YAMI-SALTY), (z) NHAA-PSECL (*Pseudomonas chlororaphis* nitrile hydratase), (T) *E. coli* apolipoprotein-*N*-acyl transferase [40,41].

high resistance to thiol reagents that strongly inhibited other nitrilases deducing therefore that the active site cysteine in this case was 'buried' and this may also be true for the *P. aeruginosa* amidase accounting for previous inconclusive results about the nature of the nucleophilic group.

With the probable exception of *E. coli* acyltransferase, all other enzymes belonging to the nitrilase group also appear to have a cysteine residue acting as an active site nucleophile. The residues were Cys^{233} for the β-ureidopropionase from *R. norvegians*, Cys^{169} for *S. cerevisiae* hypothetical protein 1, Cys^{146} for *S. aureus* hypothetical protein 5, and Cys^{119} for the *S. lugdunensis* orf5′ sequence.

Amidases fell into 4 groups, a separation confirmed by the multiple alignment of amidase sequences (data not shown), reflecting differences in their substrate specificities and metabolic roles as suggested by Hashimoto et al. [6]. Since no homol-

Enzyme	Multialignment sequences		
NRL2_ARATH	169	TPIG**KL**GAAI**C**W**E**NRM**P**LYRT	189
NRL1_ARATH	176	TPIG**KL**GAAI**C**W**E**NRM**P**LYRT	196
CYHY_GLOSO	153	.EIG**RL**GQLN**C**W**E**NMN**P**FLKS	173
CYHY_FUSLA	154	LRLAASGQLN**C**W**E**NMN**P**FLKS	174
NRL2_RHORH	154	MPFA**RL**GALN**C**W**E**HFQ**T**LTKY	174
NRL1_RHORH	160	FGFG**RV**GGLN**C**W**E**HFQ**P**LSKY	180
NRLA_ALCFA	153	TELG**RV**GALC**C**W**E**HLS**P**LSKY	173
NRLB_KLEPN	151	TSVG**RV**GALN**CA**E**N**LQ**S**LNKF	171
HYP.PROT.1	159	TAAG**KI**GGAI**C**W**E**NIM**P**LLRY	179
ALAM_RHOER	156	PKGL**KI**SLII**CDD**GNY**P**EIWR	176
ALAM_PSEAE	156	PKGM**KI**SLII**CDD**GNY**P**EIWR	176
HYP.PROT.5	136	SDGT**YV**TQLI**C**Y**D**LRF**P**ELLR	156
ORF5'	109	SNGV**KV**TQMI**C**Y**D**LRF**P**ELLR	129
BUP_RAT	223	TQFG**RI**AVNI**CYG**RHH**P**LNWL	243

Fig. 3. Multiple alignment of the active site cysteine region of nitrilases with the sequences of other proteins in the nitrilase phylogenetic group. Residues that are similar or identical in most of the sequences are shown in bold.

ogy was seen between the proposed active site region of the *P.aeruginosa* and *R. erythropolis* amidases and amidases in other groups, it seems likely that the amidases in the other groups use a different catalytic mechanism and/or different active site residues.

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