ACTIVATION OF BLAR1 PROTEIN OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*, ITS PROTEOLYTIC PROCESSING AND RECOVERY FROM INDUCTION OF RESISTANCE

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SUPPLEMENTAL DATA

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Recombinant BlaR1 expression in E. coli and membrane protein preparation. The *blaR1* gene was amplified by PCR from plamid pI258 of *S. aureus* NRS128 with Pfu Turbo DNA polymerase (Stratagene) and primers BlaREntD (5'-ata**catatg**gctaagttattaataatgagcatag) and BlaREntR (5'-at**ctcgag**ttggccatttaaaacacccatttctttt), with *Nde* I and *Xho* I sites, respectively, in bold. The resulting product contained the entire *blaR1* gene without its promoter region. The PCR product and pET-24a(+) vector were digested with *Nde* I and *Xho* I endonucleases, purified from a 1% agarose gel and ligated. The ligation mixture was transformed by electroporation into *E. coli* JM83. Colonies containing the construct were selected on LB agar containing 30 μg/mL of kanamycin. Plasmids from different colonies were isolated and sequenced. Three sequencing reactions were required to cover the entire *blaR1* gene and, apart from the T7-promoter and T7-terminator primers, we used primer BlaR_SA_seq1 (5'-gactaaaatatataatattgcatg). This construct, was designated pET-24a(+)_BlaRHis6x. *E. coli* BL21 Star™(DE3) competent cells were transformed with plasmid pET-24a(+)_BlaRHis6x, and transformants were selected on LB-kanamycin-agar plates.

A 25-ml portion of the culture was grown overnight at 37 °C, 220 rpm, in Luria Bertani (LB) medium supplemented with 50 μg/ml kanamycin. The saturated culture was centrifuged for 15 min at 2,500 *g* and the cells were then resuspended in 1 ml of fresh LB and used to inoculate 500 ml of LB supplemented with 50 μg/ml kanamycin. Cells were grown at 37 °C and 220 rpm until OD_{600} reached 1.0. Expression of the recombinant protein was induced at this point by addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and cells were grown for 5 hours at 28 °C and 220 rpm. The cultures were centrifuged for 15 min at 3,200 *g* and 4 °C, the cell pellet was washed in buffer A (100 mM Sodium Phosphate pH 7.5, 50 mM $NaHCO_3$), and centrifuged again. The cell pellets were stored overnight at -20 °C. The cell pellets from a 500-ml culture was thawed and resuspended in 20 ml Buffer A, supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM $MgCl_2$, 2.5 μg/ml DNAse I, and the cell contents were liberated by sonification in an ice-water bath (7 cycles of 1 min sonication with 1 min break in between). The extracts were centrifuged for 45 min at 18,000 *g* and 4 °C, and the supernatant was subjected to ultracentrifugation for 1 h at 130,000 *g* at the same temperature, to separate the soluble proteins (fraction designated as "Sol") from the membrane fraction (designated as "M"). The membrane fraction was resuspended in 3.3 mL of Buffer A, aliquoted and stored at - 80 ºC. A similar protocol was used to prepare membrane proteins from *S. aureus* cells, except that the cells were lysed by incubation for 30 min at 37 ºC in Lysis Buffer 1 (100 mM sodium phosphate, pH 7.5, 50 mM $NaHCO_3$, 1x Complete EDTA-free Protease Inhibitor Cocktail, 1 mM EDTA, 20 mM $MgCl_2$, 15 μg/ml DNase I, 15 μg/ml RNase A, 200 μg/mL lysostaphin), prior to sonification.

Immunoaffinity purification of BlaR1 full-length and C-terminal fragments. In order to immobilize the anti-$BlaR^S$ antibody for immunoprecipitation (anti-$BlaR^S$/Protein A resin), rabbit serum containing ~2 mg immunoglobulin (IgG) was incubated (1 h, at room temperature) with 1 ml Pierce Protein A Agarose beads (Thermo Scientific) in 10 mL binding buffer (100 mM sodium-phosphate buffer pH 7.4, 150 mM NaCl) with gentle rocking. The beads were washed twice with 0.2 M sodium-borate buffer, pH 9.0, followed by cross-linking of the antibodies to Protein A with 20 mM dimethyl pimelimidate hydrochloride (DMP) in the same buffer for 30 minutes at room temperature. After centrifugation the resin was resuspended in 0.2 M ethanolamine, pH 8.0, and incubated 2 h at room temperature. The anti-$BlaR^S$/Protein A beads were washed in binding buffer and stored at 4 °C.

In order to isolate the ~30 kDa BlaR1 fragment observed in the culture medium, the cells from 900 ml culture medium of *S. aureus* NRS128 induced for 3 h with 10 μg/ml CBAP were pelleted by centrifugation (30 minutes at 3,200 *g* at 4 °C) and the supernatant was concentrated to 15 ml using Centricon Plus-70 centrifugal filter unit (Millipore). The concentrated medium was dialyzed at 4 °C in dialysis buffer (50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 0.05% Brij

35) and centrifuged (15 min at 20,000 *g* at 4 °C). The IgG-binding proteins were removed from 5 ml supernatant by incubation with 300 µl of IgG Sepharose™ 6 Fast Flow (GE Healthcare) for 2 h at room temperature with gentle rocking. After centrifugation (10 min, 3,000 *g*) the supernatant was collected and stored at -80 °C for immunoaffinity purification of $BlaR^S$. Because the anti-$BlaR^S$ antibody can efficiently immunoprecipitate the denatured target (as determined in preliminary experiments with purified recombinant $BlaR^S$; data not shown), 1 ml of concentrated medium (stored at -80 °C) was diluted 5-fold in denaturing buffer (50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.55% SDS) and was boiled for 5 minutes. Then, the sample was supplemented with Triton X-100 (2.5% final concentration), mixed with 100 µl of anti-$BlaR^S$/Protein A resin (previously washed in the same denaturing buffer supplemented with Triton X-100) and incubated at 4 °C overnight. After a brief centrifugation, the supernatant (unbound proteins) was collected and the resin (bound proteins) was washed three times in the same buffer and once in washing buffer (50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 0.1% Nonidet-P40, and 10% glycerol). The bound proteins were eluted with 30 µl of 2x Laemmli sample buffer (non-reducing) and a small aliquot was resolved by 11% SDS-PAGE, followed by transfer to Immobilon-P membrane (Millipore) in 10 mM CAPS buffer, pH 11, and 10% methanol. $BlaR^S$ was identified by immunoblot analysis with specific anti-$BlaR^S$ antibody. All of the eluted proteins were separated on 11% SDS-PAGE, stained with Coomassie Brilliant Blue, and the protein band corresponding to the ~30 kDa BlaR1 C-terminal sensor domain fragment was cut from the gel and processed for determination of N-terminal sequence by mass spectrometry.

Following a similar procedure with anti-$BlaR^S$/Protein A resin, we isolated the 60 and 33-35 kDa BlaR1 fragments from *S. aureus* NRS128 induced for 3 h with 10 µg/ml CBAP. A 200 mg portion of the wet cell pellet was resuspended in 2 ml denaturing buffer containing 2% SDS, and was boiled for 5 minutes. The insoluble particles were removed by centrifugation (15 min at 8,000 *g* at 4 °C), the supernatant was diluted 4-fold with 50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 1x Complete EDTA-free Protease Inhibitor Cocktail (Roche), and supplemented with Triton X-100 to 2.5% final concentration. The IgG-binding proteins were removed from this solubilized cell extract with IgG Sepharose™ 6 Fast Flow. The BlaR1 fragments were isolated by immunoprecipitation with anti-$BlaR^S$/Protein A resin, separated by 11% SDS-PAGE, and identified by immunoblot analysis. The same protocol was used to immunoprecipitate BlaR1 from whole cell extracts (10 ml cultures) of NRS123, NRS70, and MRSA252 (either non-induced or CBAP-induced). NRS144 (which is devoid of the *bla* system) was used as a negative control in the immunoprecipitation procedure (incubated with CBAP). A similar protocol was applied for purification of recombinant BlaR1 expressed in *E. coli*. The bacteria were resuspended in 25 mM HEPES pH 7.5, 1 mM EDTA, 1x Complete EDTA-free Protease Inhibitor Cocktail (Roche), the cell contents were liberated by sonification, the extract was then supplemented with SDS (2% final concentration), and boiled for 5 minutes. Immunoaffinity purification of rBlaR1 from the solubilized extract was then done as described above for *S. aureus*.

Minimal inhibitory concentrations determinations in the presence of purified rBlaR^S. Based on the intensity of the BlaR1 sensor domain bands detected in the medium, compared to a standard of purified recombinant $BlaR^S$ of known concentration, we estimated that in NRS128 cultures induced for 3 h with CBAP we had 0.11 µg/ml of the shed BlaR1 sensor domain. In order to evaluate the effect of the presence of BlaR1 sensor domain on the MIC of different β-lactam compounds, we used strain NRS144, which is a methicillin-sensitive *S. aureus* (MSSA) strain that does not have the *bla* system. We determined the MICs by the broth microdilution method in BBL™ Mueller Hinton II supplemented with increasing concentrations (0-110 µg/ml) of purified recombinant $BlaR^S$.

Table S1. MIC of β-lactam antibiotics against different *S. aureus* strains, and concentrations of antibiotics used to induce the *bla* system in liquid cultures. Antibiotics used: 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP), penicillin G (PEN), ampicillin (AMP), and oxacillin (OXA).

	MIC (μg/ml)				**[antibiotic] in sub-MIC assays (μg/ml)**			
	NRS128	**NRS70**	**NRS123**	**MRSA252**	**NRS128**	**NRS70**	**NRS123**	**MRSA252**
CBAP	64	1025	1025	> 2050	10	160	160	160
PEN	64	16	16	64	20	-	5	20
AMP	64	32	128	128	20	-	40	40
OXA	0.25	32	32	256	0.08	10	10	80

Table S2. MIC of β-lactam antibiotics against *S. aureus* strain NRS144 (RN4220) in the presence and absence of added purified recombinant $BlaR^s$.

NRS144	**OXA (μg/ml)**	**PEN (μg/ml)**	**CBAP (μg/ml)**
0	0.125-0.25	0.031	64
0.011 μg/ml $rBlaR^S$	0.25	0.062	64
0.11 μg/ml $rBlaR^S$	0.25	0.062	64
1.1 μg/ml $rBlaR^S$	0.125-0.25	0.031-0.062	64
11 μg/ml $rBlaR^S$	0.5	0.25	64
110 μg/ml $rBlaR^S$	4	2	64

Figure S1. Time course of BlaI turnover and resynthesis in NRS123 **(A)**, MRSA252 **(B),** and NRS70 **(C)** whole-cells extracts. The cultures were either grown in the presence of 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP), penicillin G (PEN), ampicillin (AMP), oxacillin (OXA), or in the absence of antibiotic (non-induced, NI). In A-C: an aliquot containing 60 µg of total protein was loaded in each case. BlaI was detected by Western Blot, using antibodies specific to recombinant BlaI, and blocking with hIgG and Protein A/G Blocker. Numbers on the right indicate the position of migration of the molecular-weight markers (in kDa).

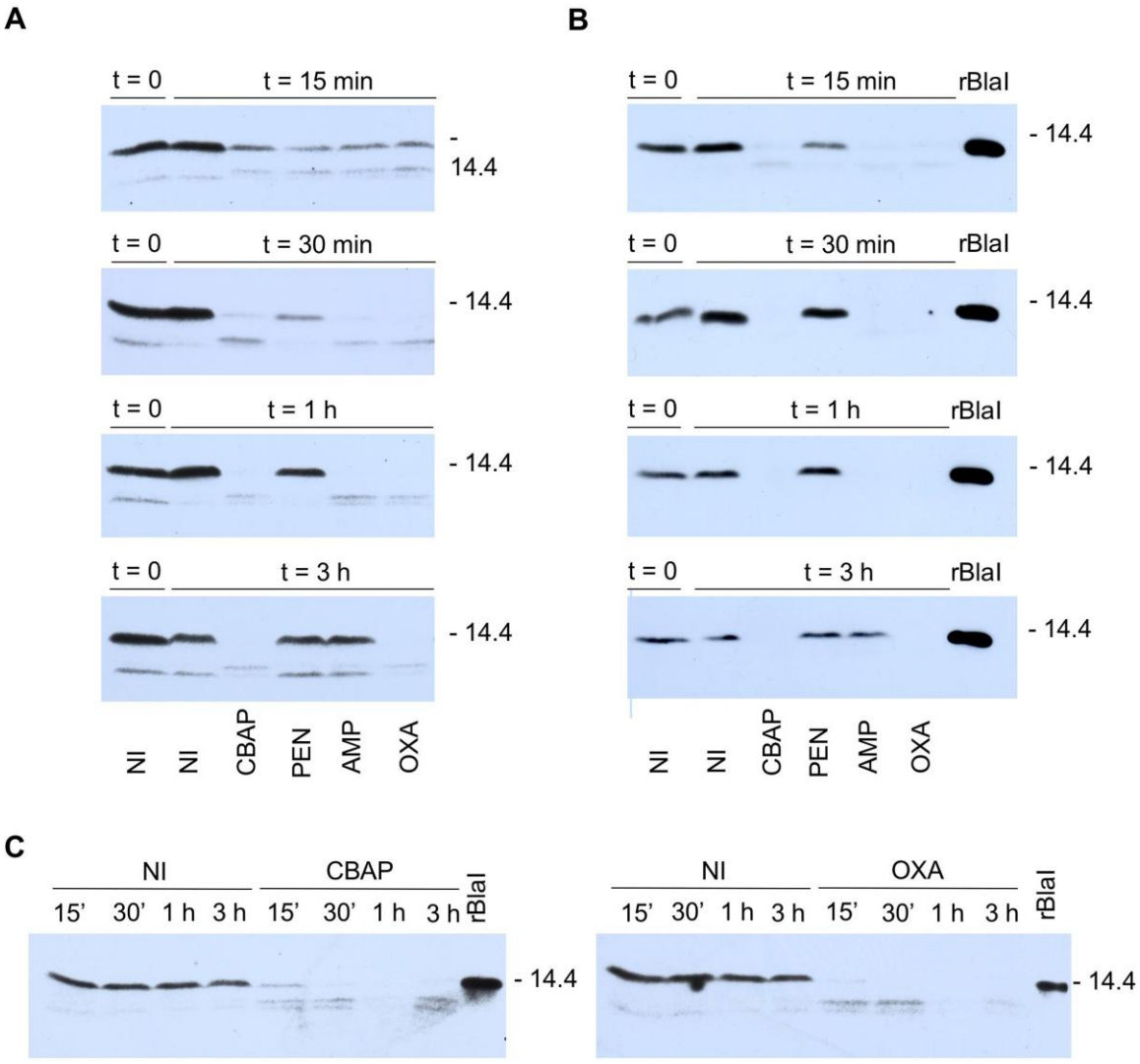


Figure S2. Time course of β-lactamase activity in the media of *S. aureus* cultures. **A.** NRS123. **B.** MRSA252. **C.** NRS70. After reaching the OD_{625} = 0.8, the cultures were incubated in the absence of antibiotic (non-induced, NI) or in the presence of different β-lactam antibiotics for 15 min (black), 30 min (white), 1 h (dark gray), and 3 h (light gray). Antibiotics used: 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP), penicillin G (PEN), ampicillin (AMP), and oxacillin (OXA).

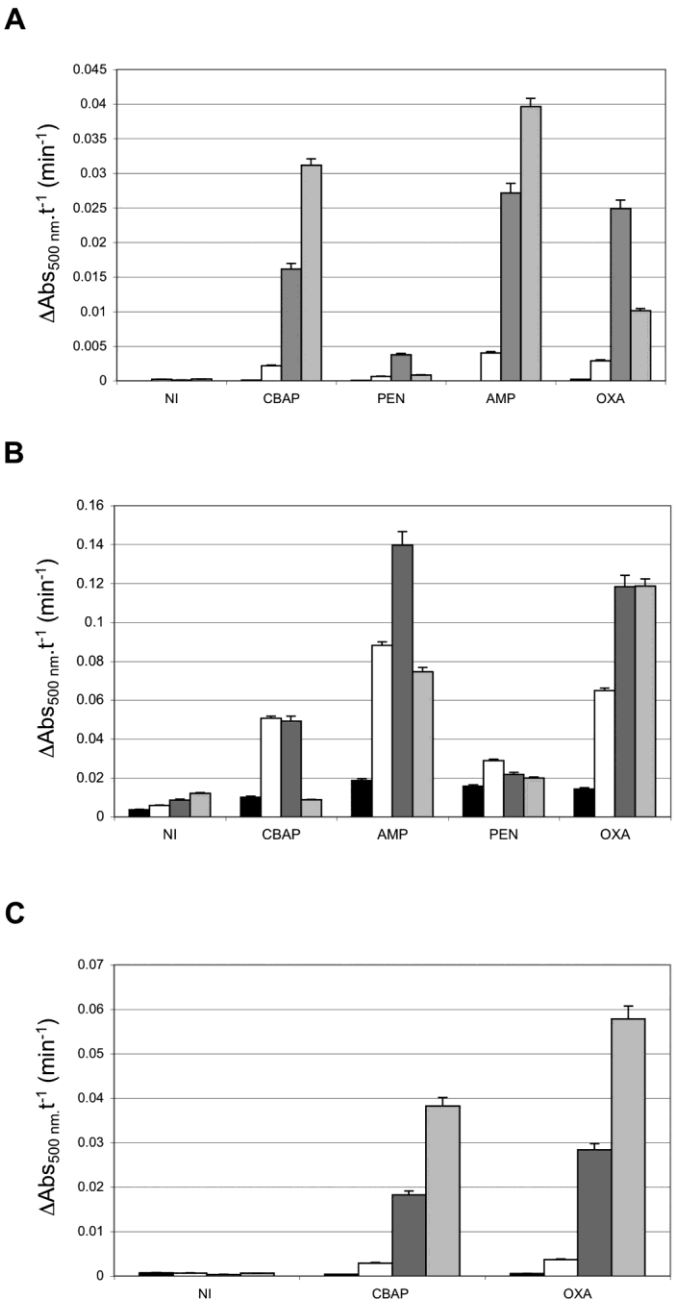


Figure S3. β-Lactamase activity measured in the media of different *S. aureus* strains, normalized according to the optical density (OD_{625}) of each culture. **A.** Non-induced (NI) samples grown to stationary phase (grown for 3 h additional hours after OD_{625} = 0.8). **B.** Comparison of activity in the media of cultures non-induced and induced for 3 h with CBAP or oxacillin (OXA). NRS128 (black), NRS123 (white), NRS70 (dark gray), and MRSA252 (light gray). **C.** Comparison of activity in the media of cultures non-induced and induced for 3 h with penicillin G (PEN) or ampicillin (AMP). NRS128 (black), NRS123 (white), and MRSA252 (light gray).

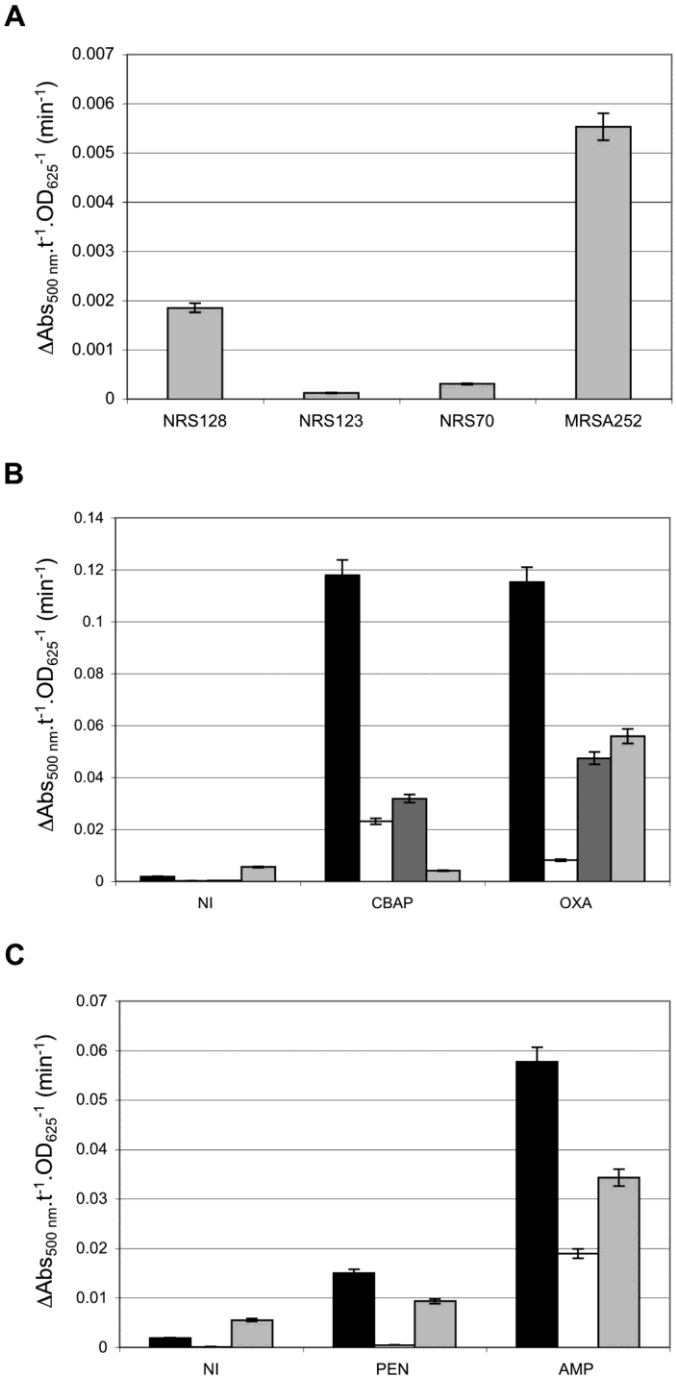


Figure S4. BlaR1 in NRS128 whole-cell extracts, non-induced (NI) and induced with different β-lactam antibiotics for different times: **A.** 15 min; **B.** 30 min; **C.** 1 h; **D.** 3 h. Same images that were given in Fig. 3B, but here showing the complete protein pattern, including the 45-70 kDa region abundant in IgG-binding proteins from *S. aureus*. The red arrows indicate the 33-35 kDa membrane-anchored C-terminal BlaR1 fragment. The band at ~28 kDa seen in samples from non-induced cultures in at t = 0 and in all samples from cultures induced for 15 min, 30 min, and 1h, is likely an IgG-binding proteins, since it was also seen in Western blots developed with several other rabbit antibodies of different specificities (data not shown). The Western blot was carried out using anti-BlaR^S specific primary antibody, anti-rabbit-HRP as secondary antibody, and blocking the membrane with hIgG and Protein A/G blocker. Antibiotics used: 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP), penicillin G (PEN), ampicillin (AMP), and oxacillin (OXA). Numbers on the right indicate the position of migration of the molecular-weight markers (in kDa).

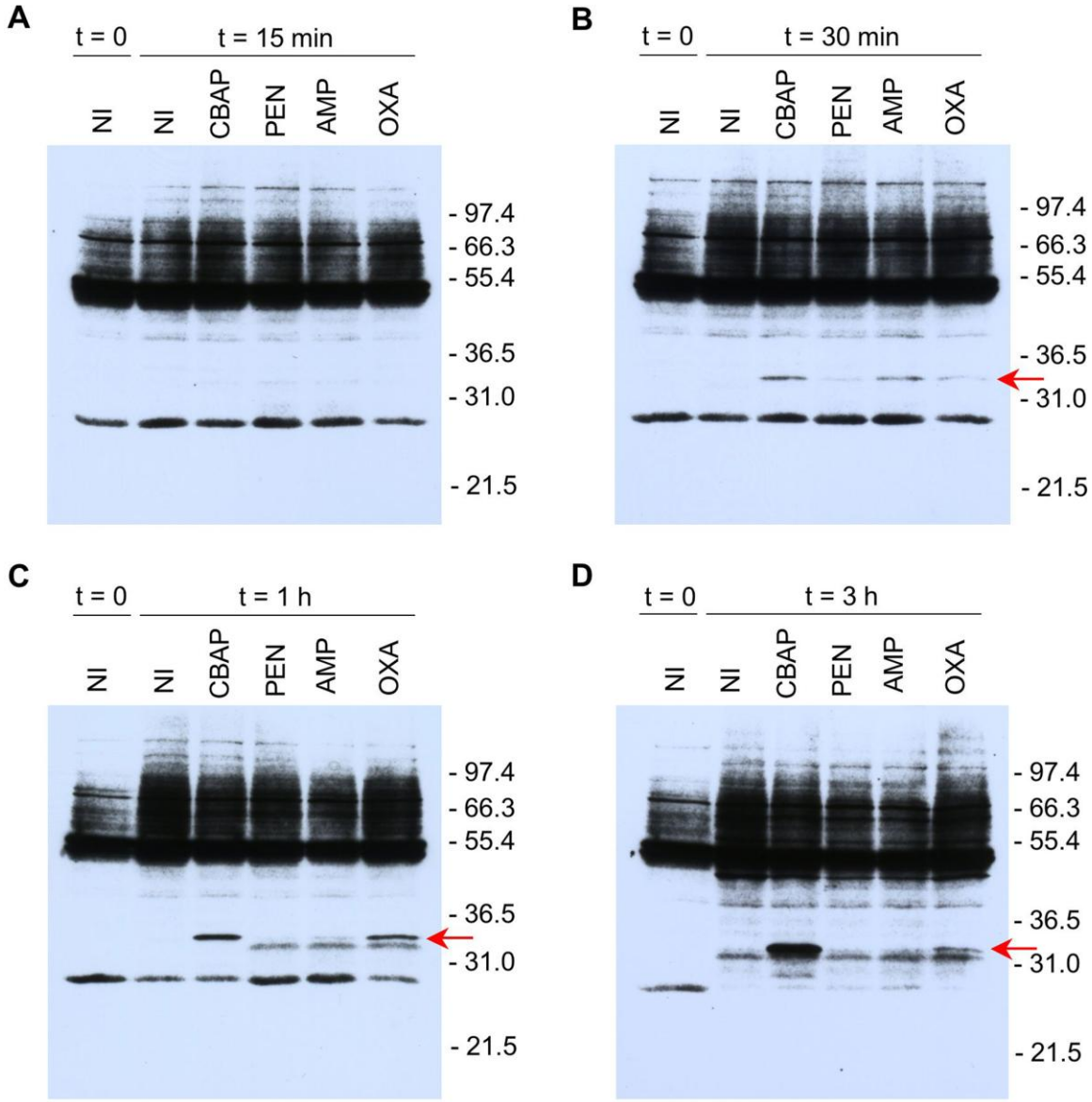


Figure S5. A. Western Blot of whole-cell extracts (80-µg portion of total protein) of strains NRS144, NRS100 (COL), and NRS128, non-induced and induced for 3 hours or overnight with CBAP. The anti-BlaR^S antibody was used for Western blot analysis using the One_Hour IP-Western bot Kit. Strain NRS144 and NRS100 do not present the *bla* operon. Strain NRS100 expresses PBP2a constitutively, since it has a deletion of the *mecI* gene. It also has a deletion of the C-terminal of the *mecR1* gene. The ~66 and ~55 kDa bands observed in whole-cell extracts are likely IgG-binding proteins, since they are present in strains with or without the *blaR1* gene. NI: non-induced, WCE: whole cell extracts. **B.** Same membrane used for Western blot in A, stripped and blotted again using the primary antibody specific to BlaR^S, and the protocol that involves blocking with hIgG and Protein A/G Blocker. Note the increase of non-specific signals comparison to panel A, and the increased sensitivity for detection of the 33-35 kDa BlaR1 band in CBAP-induced NRS128 whole-cell extract. In A and B: WCE: whole-cell extract; ON, over-night induction; 3h, induction for 3h. Numbers on the right indicate the position of migration of the molecular-weight markers (in kDa).

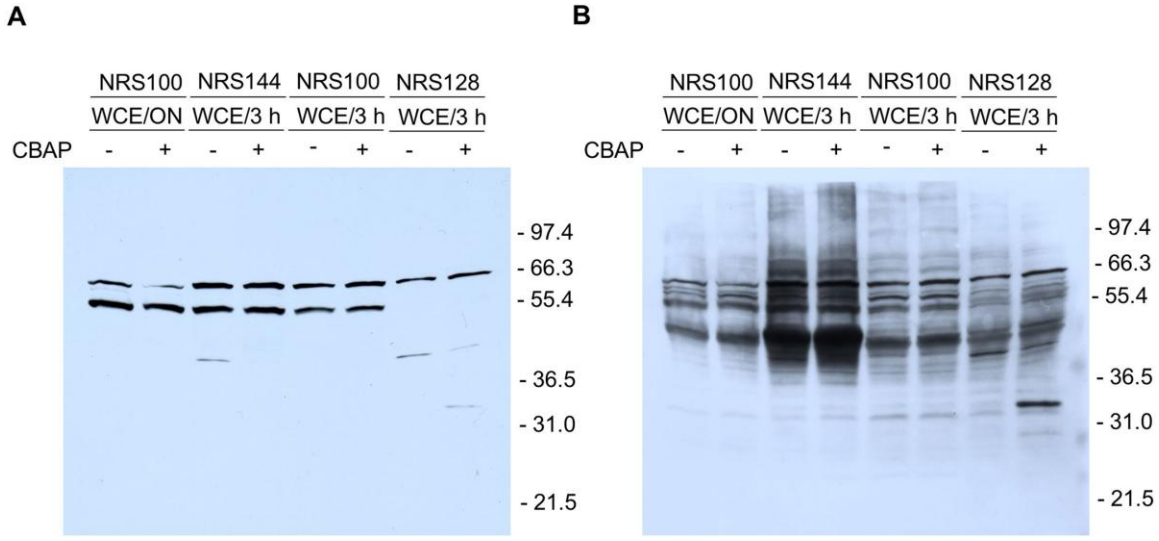


Figure S6. Tryptic coverage for rBlaR1 peptides identified from in-gel digestion of the regions around 60 (A) and 33-35 kDa (B) from whole-cell extracts of BlaR1 expressed in *E. coli*. Peptides from both the N- and C-terminal ends of rBlaR1 were present in both 60 kDa and 33-35 kDa regions. Red bold indicates an MS/MS sequenced peptide with high confidence; black bold indicates precursor mass evidence only. Both Samples identified rBlaR1 with > 99.9% confidence.

A. rBlaR1 in *E. coli* whole-cell extract, ~60 kDa region in the gel.

MAKLLIMSIVSFCFIFLLLLFFRYILKRYFNYMLNYKVWYLTLLAGLIPFIPIKFSLFKF
NNVNNQAPTVESKSHDLNHNINTTKPIQEFATDIHKFNWDSIDNICTVIWIVLVIILSFK
FLKALLYLKYLK**KQSLYLNENEKNKIDTILFNHQYKKNIVIRKAETIQSPITFWYGKYII**
LIPSSYFKSVIDKRLKYIILHEYAHAKNRDTLHLIIFNIFSIIMSYNPLVHIVKRK IIHD
NEVEADRFVLNNINKNEFKTYAESIMDSVLNVPFFNKNILSHSFNGKKSLLKRRLINIKE
ANLKKQSKLILIFICIFTFLLMVIQSQFLMGQSITDYNYK**KPLHNDYQILDK**SKIFGSNS
GSFVMYSMKK**DKYYIYNEKESRKRYSPNSTYKIYLAMFGLDRHIINDENSRMSWNHKHYP**
FDAWNKEQDLNTAMQNSVN**WYFERISDQIPKNYTATQLK**QLNYGNK**NLGSYKSYWMEDSL**
KISNLEQVIVFKNMMEQNNHFSKKAKNQLSSSLLIKKNEKYELYGKTGTGIVNGKYNNGW
FVGYVITNHDKYY**FATHLSDGKPSGKNAELISEKILKEMGVLNGQ**

B. rBlaR1 in *E. coli* whole-cell extract, ~33-35 kDa region in the gel.

MAK**LLIMSIVSFCFIFLLLLFFRY**ILKRYFNYMLNYKVWYLTLLAGLIPFI**PIKFSLFKF**
NNVNNQAPTVESKSHDLNHNINTTKPIQEFATDIHKFNWDSIDNICTVIWIVLVIILSFK
FLKALLYLKYLKK**QSLYLNENEKNKIDTILFNHQYKKNIVIR**K**AETIQSPITFWYGKYII**
LIPSSYFKSVIDKRLKYIILHEYAHAKNRDTLHLIIFNIFSIIM**SYNPLVHIVKRKIIHD**
NEVEADRFVLNNINKNEFKTYAESIMDSVLNVPFFNKNILSHSFNGKKSLLKRRLINIKE
ANLKKQSKLILIFICIF**TFLLMVIQSQFLM**GQSI**TDYNYKKPLHNDYQILDK**SK**IFGSNS**
GSFVMYSMKKDKYYIYNEKESRK**RYSPNSTYKIYLAMFGLDRHIINDENSRMSWNHKHYP**
FDAWNKEQDLNTAMQNSVNWYFER**ISDQIPKNYTATQLKQLNYGNKNLGSYK**SYWMEDSL
K**ISNLEQVIVFKNMMEQNNHFSKKAKNQLSSSLLIKKNEKYELYGKTGTGIVNGK**YNNGW
FVGYVITNHDKYYF**ATHLSDGKPSGKNAELISEKILKEMGVLNGQ**

Figure S7. Annotated MS/MS spectra of two example BlaR1 peptides identified in whole-cell extract of rBlaR1 expressed in *E. coli*: F^{60}NNVNNQAPTVESK, a peptide from the predicted loop 2 (which follows the second predicted membrane-spanning region), and I^{482}SNLEQVIVFK, a peptide from the extracellular-sensor domain. **A.** Peptides from the 60 (± 5) kDa region. **B**. Peptides from the 34 (± 5) kDa region.

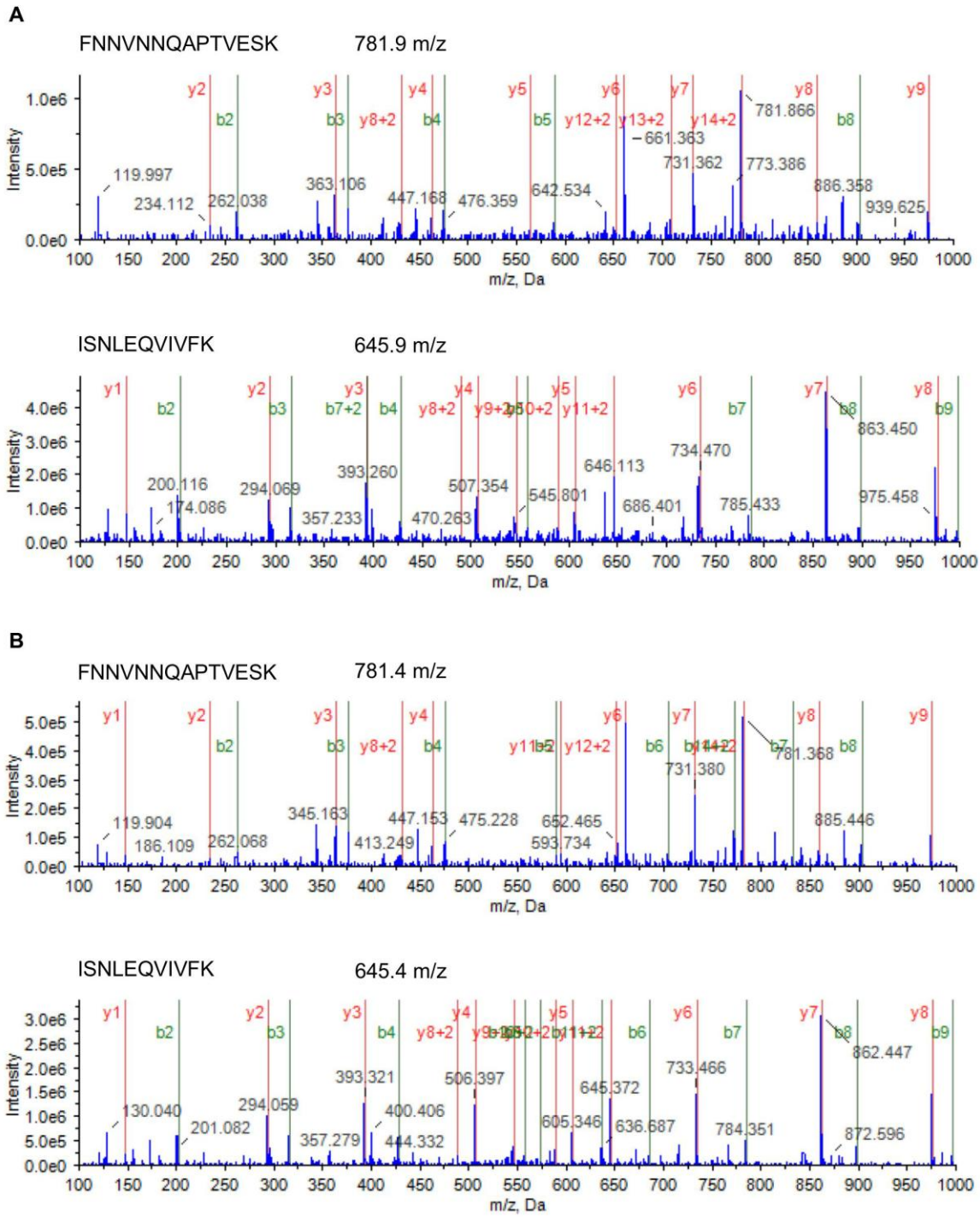


Figure S8. A. Time course of accumulation of BlaR1 species in *S. aureus* strains NRS123, MRSA252, and NRS70 after activation of the *bla* system by β-lactams. **A.** NRS123. **B.** MRSA252. **C.** NRS70. The samples correspond to whole-cell extracts of non-induced cultures (NI) or induced for the indicated time with CBAP, penicillin, ampicillin, and oxacilin. The Western blot was carried out using anti-BlaR^S specific primary antibody, anti-rabbit-HRP as secondary antibody, and blocking the membrane with hIgG and Protein A/G blocker. The 33-35 kDa BlaR1 fragment shown in Fig. 3 for NRS128 could only be detected clearly in MRSA252 extracts after 1 hour induction with all four antibiotics. It could not be detected in NRS123 and NRS70 whole-cell extracts. Antibiotics used: 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP), penicillin G (PEN), ampicillin (AMP), and oxacillin (OXA). Numbers on the right indicate the position of migration of the molecular-weight markers (in kDa).

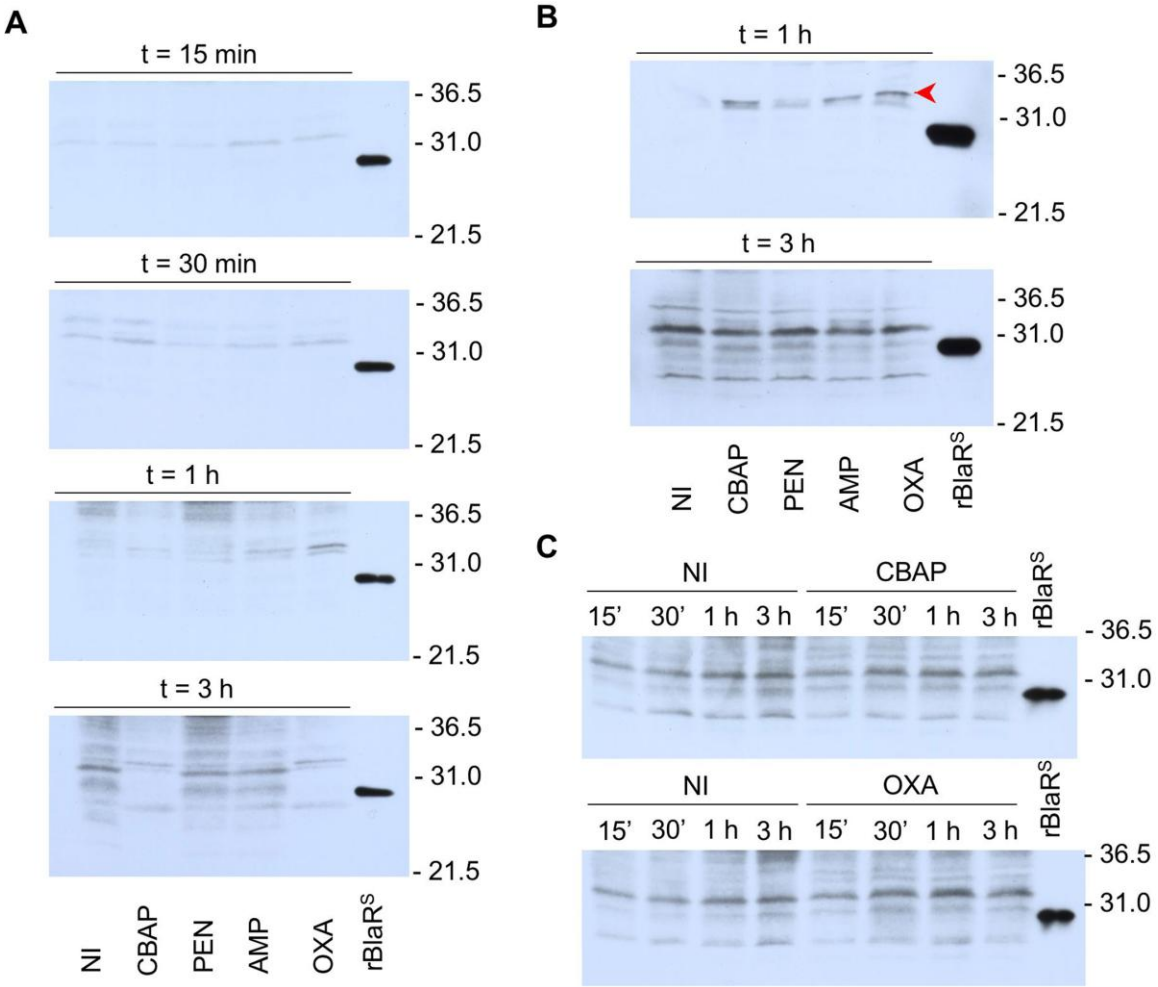


Figure S9. MRM transitions for BlaR1 peptides of the 33-35 kDa band excised from SDS-PAGE, showing the N-terminal F^{284}NGKKLL peptide and a sensor-domain peptide (Y^{339}KKPLHNDY) for the proteins expressed in *E. coli* and in CBAP-induced NRS128. **A**. Recombinant BlaR1 (rBlaR1) immunoprecipitated from *E. coli*, excised and in-gel digested with pepsin. **B**. Negative control (66 kDa molecular-weight marker (BSA) digested with pepsin), run in between samples A and C. **C**. BlaR1 immunoprecipitated from CBAP-induced *S. aureus* NRS128, excised and in-gel digested with pepsin. Retention times were confirmed from MS/MS spectra acquired on rBlaR1 digests with pepsin (See Fig. S10 for spectra). Peptide transitions used were: F^{284}NGKKSLL [453.77→645.4(2y6),759.5,(2y7),662.4(2b6)], (S/N C = 42:1@3σ); Y^{339}KKPLHNDY [589.3→886.4(2y7),758.3,(2y6),767.5(2b6)], (S/N = C = 8:1@3σ). A quantitative analysis of the intensity of the signal of the F^{284}NGKKSLL peptide compared to the intensity of the sensor-domain peptide Y^{339}KKPLHNDY showed a similar ratio of the intensities of the peptides in the *E. coli* and NRS128 samples (1.9 vs 1.8, respectively). This supports that the 33-35 kDa BlaR1 fragment immunoprecipitated from CBAP-induced NRS128 is a homogeneous protein, which has the same N-terminus as the protein expressed in *E. coli.* Also, the differences in apparent mass in SDS-PAGE of the 33-35 kDa BlaR1 bands in *E. coli* and NRS128 are also consistent with only a 1-kDa mass difference, due to the C-terminal His6x-tag in the bigger BlaR1 fragment of the protein expressed in *E. coli*.

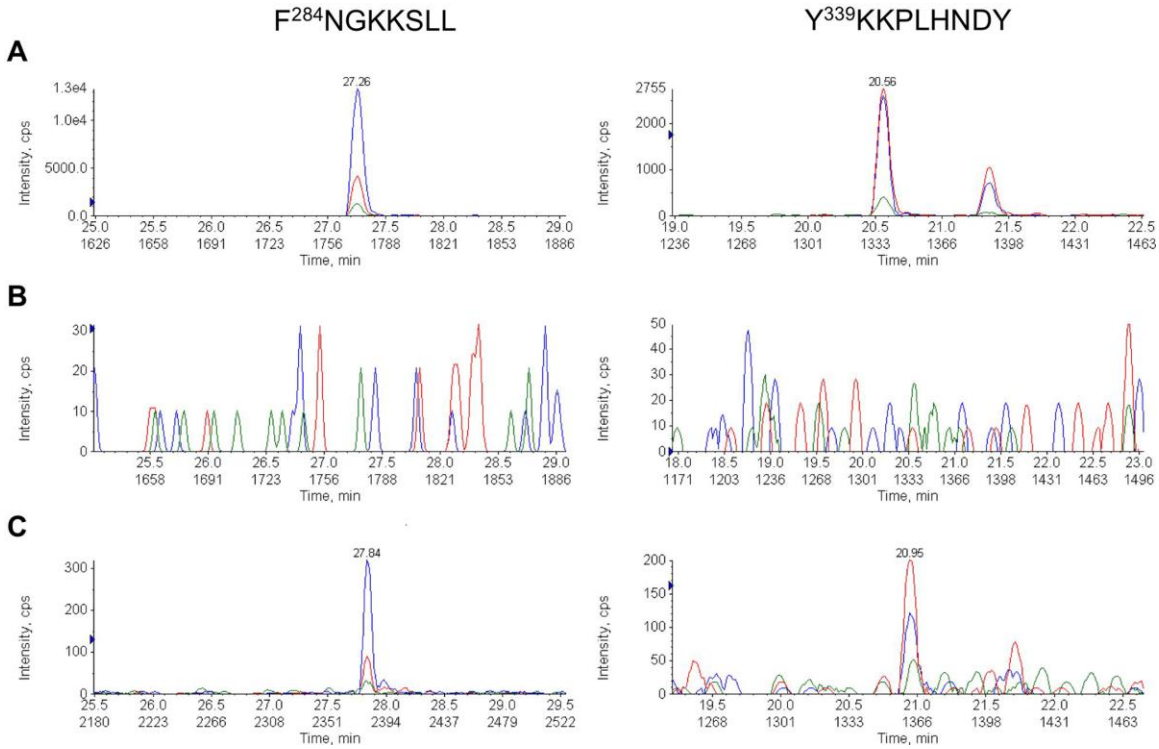
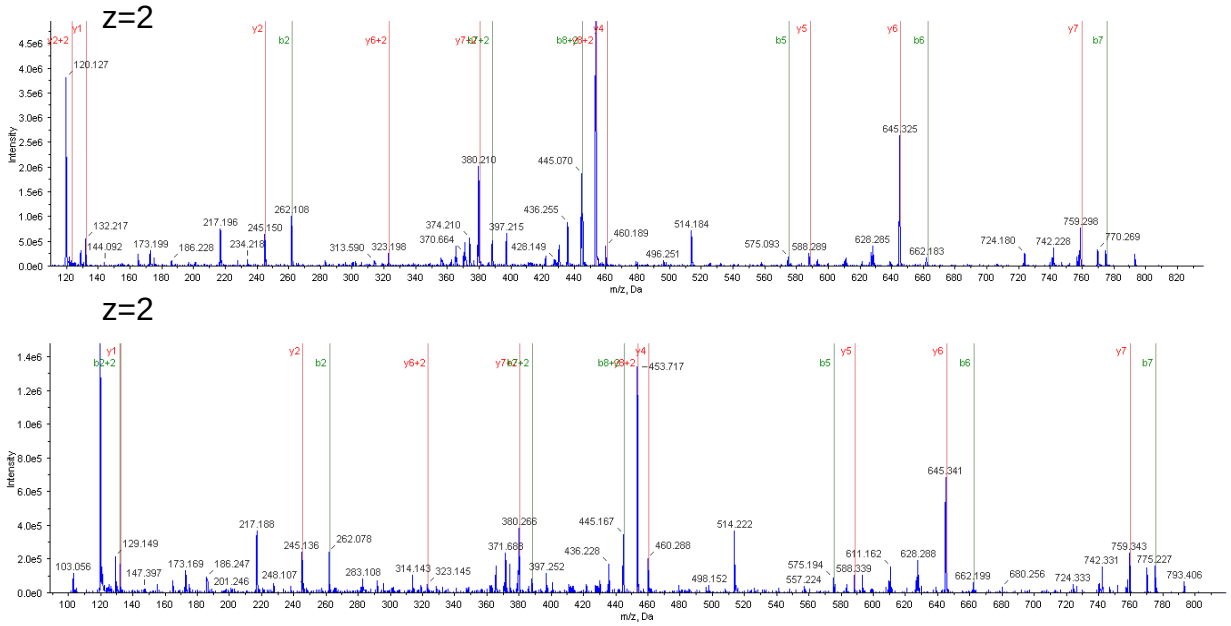
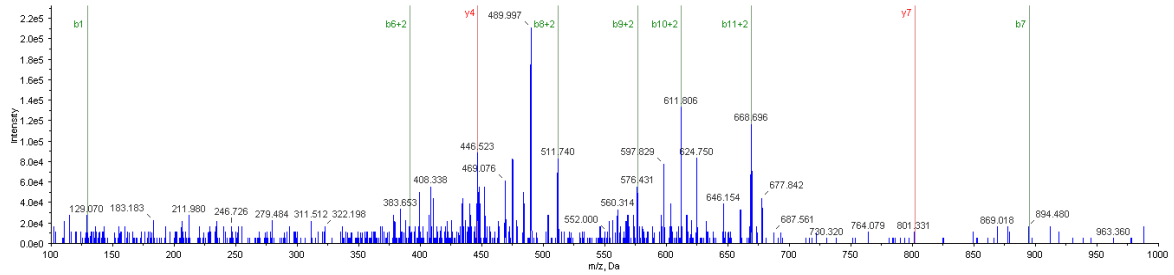


Figure S10. MS/MS Spectra from peptides F^{284}NGKKSLL (m/z 453.77^{2+}), KR^{293}RLINIKEANL (m/z 489.98^{3+}), and Y^{339}KKPLHNDY (*m/z* 589.3^{+2}), which were obtained from the in-gel pepsin digested 33-35 kDa band of immunoprecipitated recombinant BlaR1 expressed in *E. coli*.

F^{284}NGKKSLL



KR^{293}RLINIKEANL



Y^{339}KKPLHNDY

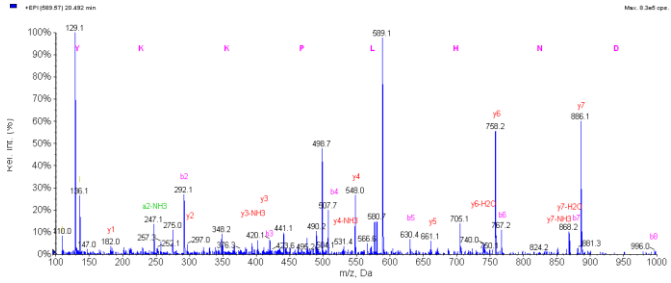


Figure S11. MS/MS Spectra the peptide F[60]NNVNNQAPTVESK (*m/z* 781.88 $^{2+}$,521.26^{3+}), which was obtained from an in-gel trypsin digested 60 kDa band of immunoprecipitated recombinant BlaR1 expressed in *E. coli*.

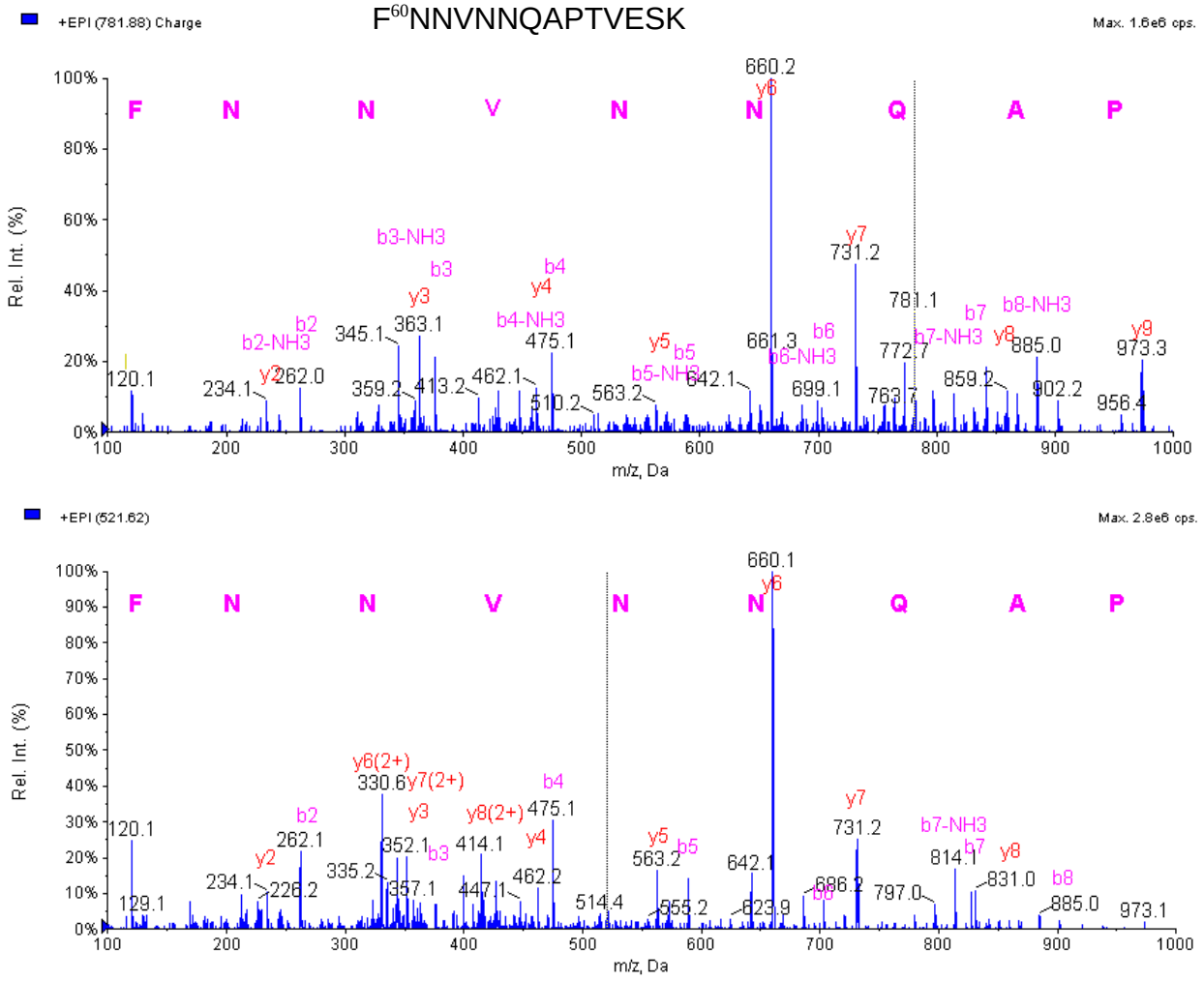
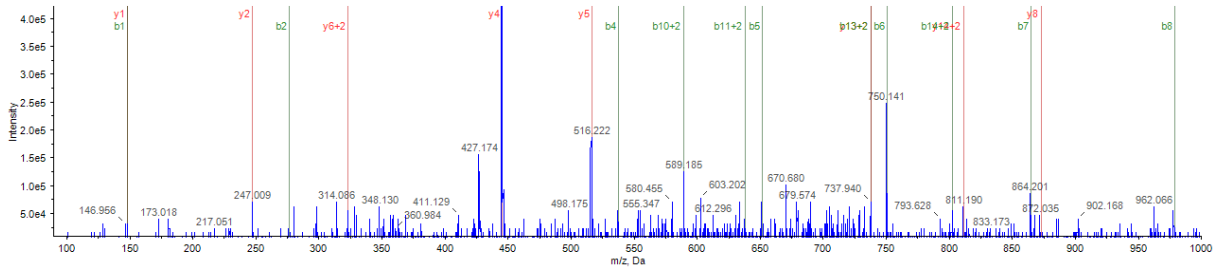


Figure S12. MS/MS Spectra from peptides F[58]KFNNVNNQAPTVESK (*m/z* 918.97^{2+}) and I[48]PFIPIKF (*m/z* 487.81^{2+}), which were obtained from the in-gel pepsin digested 60 kDa band of immunoprecipitated recombinant BlaR1 expressed in *E. coli*.

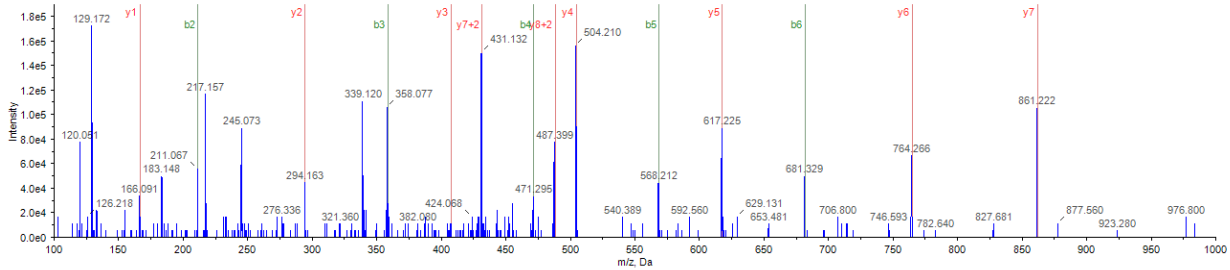
z=2

F[60]KFNNVNNQAPTVE



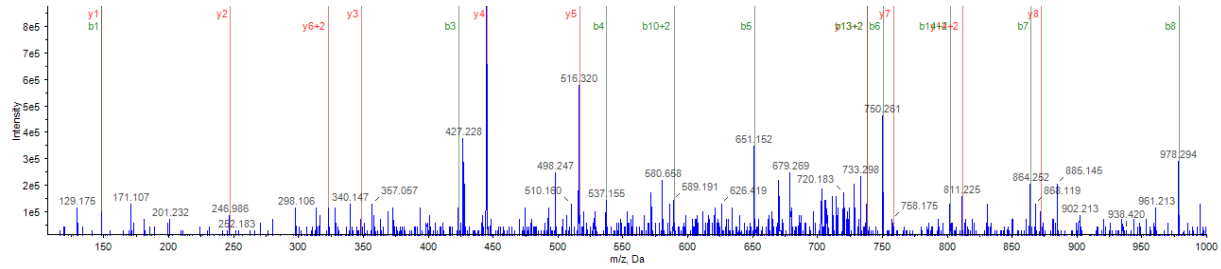
I[48]PFIPIKF

z=2



z=2

F[60]KFNNVNNQAPTVE Technical Replicate



z=2

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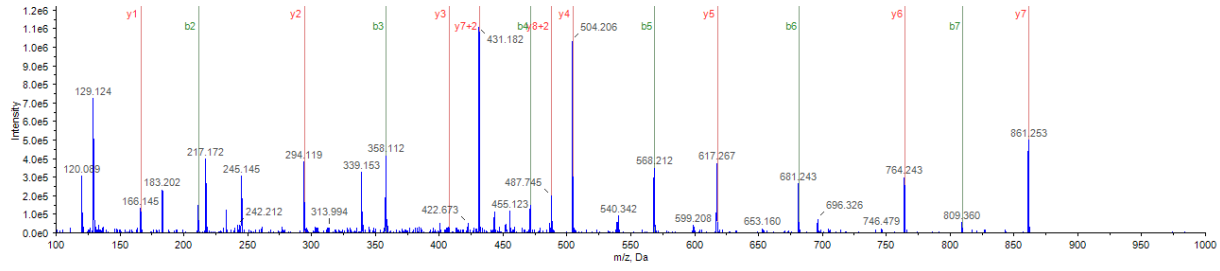

Figure S13. MRM transitions for BlaR1 peptides obtained from in-gel trypsin digestion of the ~60 kDa BlaR1 band of immunoprecipitated samples. **A.** Blank. **B.** Recombinant BlaR1 expressed in *E. coli*. **C.** BlaR1 from CBAP-induced NRS128. Peptide transitions used were (From Left to Right): F^{248}VLNNINK [481.3 → 814.5(2y7), 715.4(2y6), 602.3(2y5]), N^{507}QLSSSLLIK [551.8 → 860.46(2y8), 747.46(2y7), 600.43(2y6)], I^{482}SNLEQVIVFK [645.38 → 862.5(2y7), 733.5(2y6), 975.6(2y8)], F^{60}NNVNNQAPTVESK [781.4 → 660.4(2y6), 731.4(2y7), 859.5(2y8), 1186.6(2y11).]. F^{248}VLNNINK is specific to the predicted cytoplasmic domain of BlaR1, N^{507}QLSSSLLIK and I^{482}SNLEQVIVFK are specific to the C-terminal sensor domain, and F^{60}NNVNNQAPTVESK is the first N-terminal tryptic fragment detected from full-length BlaR1.

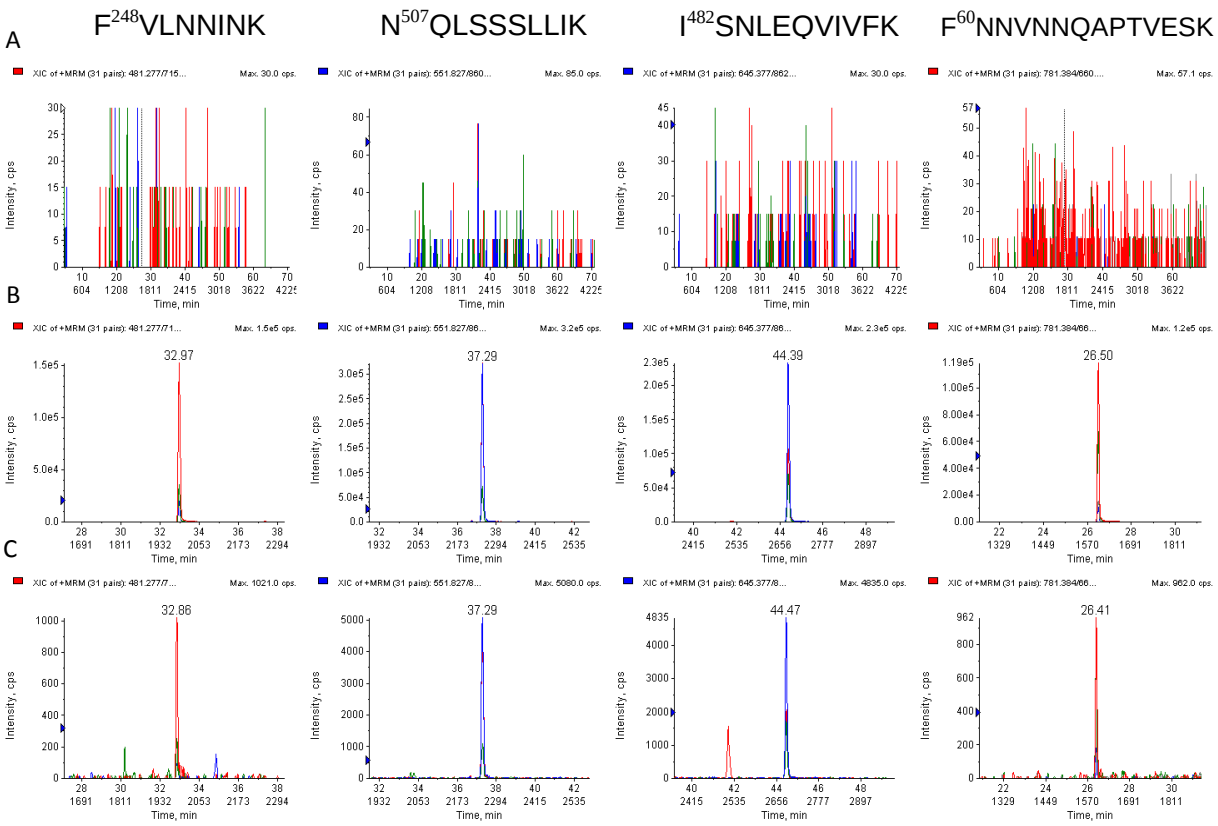

Figure S14. The sensor domain of BlaR1 is also shed in antibiotic-induced *S. aureus* MRSA252, but not in NRS123 or NRS70. **A.** NRS123. **B.** MRSA252. **C.** NRS70. A-C: detection of the C-terminal domain of BlaR1 in the media of the *S. aureus* NRS128 culture after induction with different β-lactam antibiotics (15 min, 30 min, 1h, and 3h). Antibiotics used: 2-(2'-carboxyphenyl)-benzoyl-6-aminopenicillanic acid (CBAP), penicillin G (PEN), ampicillin (AMP), and oxacillin (OXA). **D.** Comparison of the level of BlaR1 sensor domain shed to the media in strains NRS128 and MRSA252, showing the higher concentration in the media of strain NRS128 (estimated at 0.11 μg/ml $BlaR^S$ in NRS128, and 0.06 μg/ml $BlaR^S$ in MRSA252). The BlaR1 species was detected by Western blot either using anti-$BlaR^S$ specific primary antibody, anti-rabbit-HRP as secondary antibody, and blocking the membrane with hIgG and Protein A/G blocker (A and C), or using anti-$BlaR^S$ specific antibody and the ONE-HOUR IP-Western™ Kit (B and D). Numbers on the right indicate the position of migration of the molecular-weight markers (in kDa).

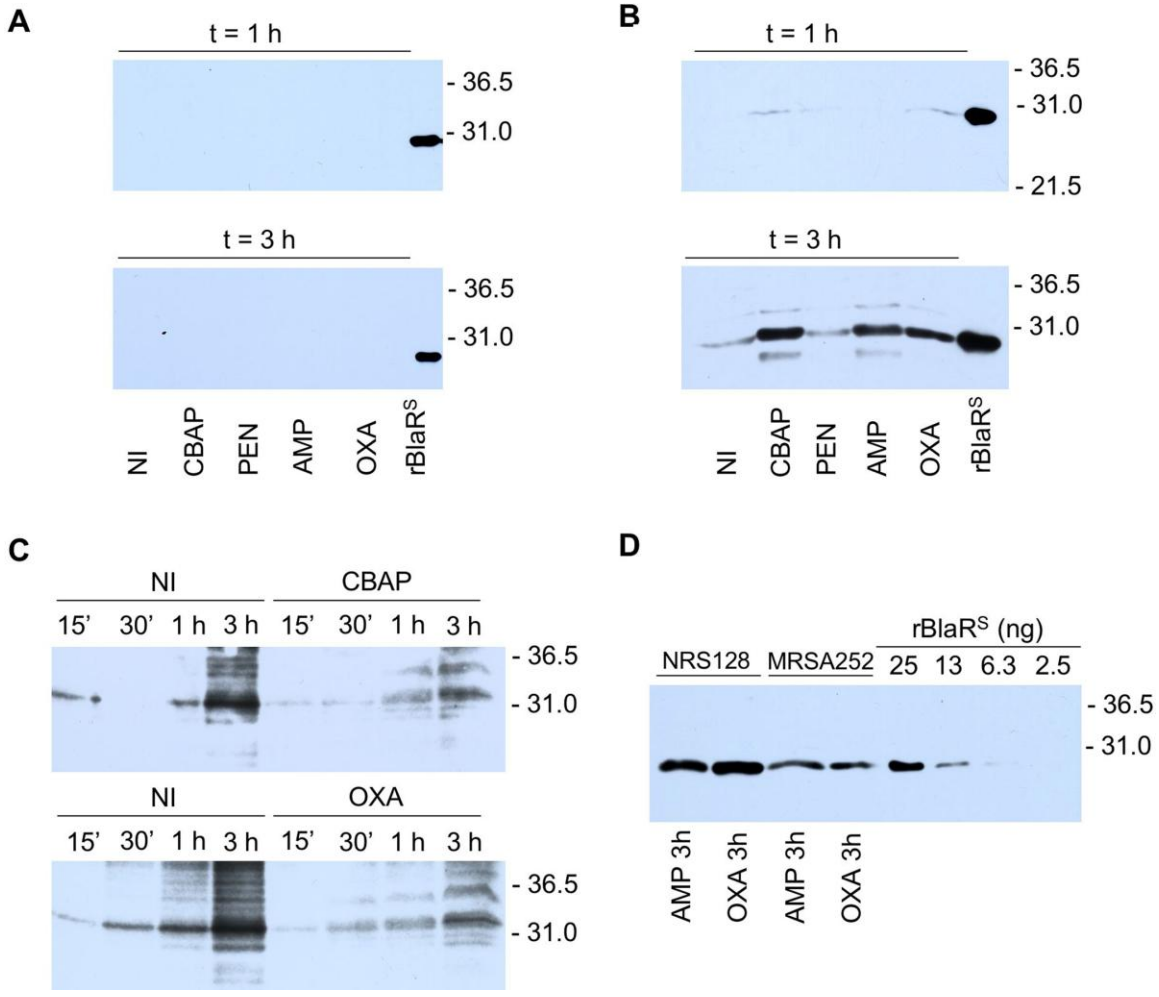

Figure S15. Tryptic BlaR1 peptides identified from the BlaR1 fragment isolated from the media of NRS128 culture induced with CBAP for 3 h by LC/MS/MS (highlighted in red). The N-terminal peptide, Q^{332}SITDYNYK, is the product of a non-trypsin cleavage at its N-terminus (cleavage after Gly-331), indicating this is the N-terminus of the BlaR1 fragment. Peptides identified from this fragment by LC/MS/MS are highlighted in red. The N-terminal residue (Gln-332) is also highlighted in boldface.

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        10         20         30         40         50         60
MAKLLIMSIV SFCFIFLLLL FFRYILKRYF NYMLNYKVWY LTLLAGLIPF IPIKFSLFKF
        70         80         90        100        110        120
NNVNNQAPTV ESKSHDLNHN INTTKPIQEF ATDIHKFNWD SIDNISTVIW IVLVIILSFK
       130        140        150        160        170        180
FLKALLYLKY LKKQSLYLNE NEKNKIDTIL FNHQYKKNIV IRKAETIQSP ITFWYGKYII
       190        200        210        220        230        240
LIPSSYFKSV IDKRLKYIIL HEYAHAKNRD TLHLIIFNIF SIIMSYNPLV HIVKRKIIHD
       250        260        270        280        290        300
NEVEADRFVL NNINKNEFKT YAESIMDSVL NVPFFNKNIL SHSFNGKKSL LKRRLINIKE
       310        320        330        340        350        360
ANLKKQSKLI LIFICIFTFL LMVIQSQFLM GQSITDYNYK KPLHNDYQIL DKSKIFGSNS
       370        380        390        400        410        420
GSFVMYSMKK DKYYIYNEKE SRKRYSPNST YKIYLAMFGL DRHIINDENS RMSWNHKHYP
       430        440        450        460        470        480
FDAWNKEQDL NTAMQNSVNW YFERISDQIP KNYTATQLKQ LNYGNKNLGS YKSYWMEDSL
       490        500        510        520        530        540
KISNLEQVIV FKNMMEQNNH FSKKAKNQLS SSLLIKKNEK YELYGKTGTG IVNGKYNNGW
       550        560        570        580
FVGYVITNHD KYYFATHLSD GKPSGKNAEL ISEKILKEMG VLNGQ
```

Figure S16. Annotated MS/MS spectra from the N-terminal peptide detected from the BlaR1 fragment isolated from the media of NRS128 culture induced with CBAP for 3 h. Top and bottom represent replicate injections of the same in-gel digest. **A.** (Top & bottom) is an XIC for the *m*/*z* of the [M+2H]+ precursor ion at ~566.1. **B**. (Top & bottom) are the annotated MS/MS spectra of the sequence Q^{332}SITDYNYK.