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1 # Production of Plantaricin NC8 by *Lactobacillus*
2 # *plantarum* NC8 is Induced in the Presence of
3 # Different Types of Gram-positive Bacteria

4

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Abstract

Lactobacillus plantarum NC8 was shown to produce plantaricin NC8 (PLNC8), a recently purified and genetically characterized inducible class IIb bacteriocin, only when it was co-cultured with other Gram-positive bacteria. Among 82 strains belonging to the genera *Bacillus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Listeria*, *Pediococcus*, *Staphylococcus* and *Streptococcus*, forty-one of them where shown to induce PLNC8 production in *L. plantarum* NC8. There was apparently no relationship between sensitivity of strains and their ability to induce the bacteriocin thus indicating that both the inducer and sensitive phenotypes may not be linked. In some instances, the induction was promoted by both living and heat-killed cells of the inducing bacteria. However, no PLNC8-inducing activity was found in their respective cell-free, pure culture supernatants. Inducer strains promoted also production of a PLNC8-autoinducing activity by *L. plantarum* NC8, which was found only in the cell-free co-culture supernatants showing inhibitory activity. This PLNC8-autoinducing activity was shown to be diffusible, heat resistant and of proteinaceous nature, being different from the bacteriocin itself. Our results suggest that the presence of specific Gram-positive bacteria act as an environmental stimulus activates PLNC8 production by *L. plantarum* NC8 as well as a PLNC8-autoinducing activity which triggers or maintains bacteriocin production in the absence of inducing cells.

Key words Autoinduction · Induction · *L. plantarum* · Plantaricin NC8

1 **Introduction**

2

3 Bacteriocins produced by lactic acid bacteria (LAB) are peptides or proteins that inhibit the
4 growth of strains and species usually related to or of species sharing similar nutritive
5 requirements with the bacteriocin-producing bacteria (de Vuyst 1994; de Vuyst and
6 Vandamme 1994; Jack et al. 1993; Klaenhammer 1993; Tagg et al. 1976). Therefore,
7 bacteriocin production seems to be a defence mechanism directed to compete for nutrients
8 against other bacteria in the same environment (Barefoot and Grinstead 1993; Dykes 1995;
9 Dykes and Hastings 1997; Riley 1998).

10 Several reports have shown that production of bacteriocins by some Gram-positive
11 bacteria is a regulated process. Production of nisin by *Lactococcus lactis* and subtilin by
12 *Bacillus subtilis*, respectively, two modified class I bacteriocins, is regulated by a cell-
13 density dependent quorum sensing mechanism (Gutowski-Ecked et al. 1994; Kleerebezem
14 et al. 1997; Kleerebezem and Quadri 2001; Klein et al. 1993; Kuipers et al. 1995; Kuipers
15 et al. 1998). In these cases, the bacteriocins are also autoinducers. Production of several
16 class II bacteriocins such as sakacin P by *Lactobacillus sake* LTH673 (Brurberg et al.
17 1997; Eisink et al. 1996), enterocins A and B by *Enterococcus faecium* CTC 492 (Nilsen et
18 al. 1997), plantaricin ABP-118 by *L. salivarius* subsp. *salivarius* UCC118 (Flynn et al.
19 2002), plantaricins E/F and J/K by *Lactobacillus plantarum* C11 (Diep et al. 1994, 1995,
20 and 1996; Moll et al. 1999), sakacin A by *L. sakei* Lb706 (Axelsson and Holck 1995; Diep
21 et al. 2000), and carnobacteriocins A and B2 by *Carnobacterium piscicola* LV17 (Franz et
22 al. 2000; Kleerebezem et al. 2001; Quadri et al. 1997; Saucier et al. 1995; Saucier et al.
23 1997) have been shown to be regulated via a quorum sensing mechanism mediated by
24 peptide pheromones or autoinducer peptides. However, in most of them, the quorum
25 sensing mechanism is not sufficient to maintain bacteriocin production in highly diluted

1 cultures (Kleerebezem et al. 1997; Kleerebezem and Quadri 2001; Nes et al. 1996; Nes and
2 Eijsink 1999). Since concentration of the peptide pheromones in those diluted cultures is
3 not sufficient to trigger bacteriocin production, the involvement of other unknown factors
4 can be assumed. Environmental factors such as pH, temperature, and other growth
5 conditions have been proposed to play an important role in regulation of bacteriocin
6 production (Diep et al. 1995; Diep et al. 2000; Kleerebezem et al. 1997; Kleerebezem and
7 Quadri 2001; Nes et al. 1996; Nes and Eijsink 1999; Nilsen et al. 1997; Saucier et al.
8 1995). Nevertheless, little or nothing is known about how these factors interact with the
9 regulatory systems that control bacteriocin production.

10 The presence of competing microorganisms has been reported as an environmental
11 factor affecting production of bacteriocins by some LAB (Barefoot et al. 1994a and 1994b;
12 Maldonado et al. 2003; Sip et al., 1998). Thus, production of lactacin B and divercin by
13 *Lactobacillus acidophilus* N2 and *Carnobacterium divergens* AS7, respectively, is
14 enhanced in the presence of the indicator strains *L. delbrueckii* ATCC 4797 (Barefoot et al.
15 1994a and 1994b) and *C. piscicola* NCDO 2765 (Sip et al. 1998), respectively. More
16 recently, the inducible bacteriocin plantaricin NC8 (PLNC8) produced by *L. plantarum*
17 NC8 has been purified and genetically characterized (Maldonado et al. 2003). This
18 bacteriocin consists of two distinct peptides, named PLNC8α and PLNC8β, whose
19 complementary action is necessary for full PLNC8 activity. In this work we show that, in
20 contrast to previously reported induced bacteriocins, PLNC8 is produced only after co-
21 culture with certain Gram-positive bacteria. As a response to the presence of the other
22 bacteria, a PLNC8-autoinducing activity which is most probably involved in the regulation
23 of PLNC8 production was also produced by *L. plantarum* NC8.
24

Materials and methods

Bacterial strains and media

The plantaricin NC8-producer *Lactobacillus plantarum* NC8, a strain originally isolated from grass silage (Aukrust and Blom 1992), has been described previously (Maldonado et al. 2003). It was maintained at –80ºC as frozen stocks in de Man, Rogosa and Sharpe medium (MRS; Oxoid, Basingstoke, Hampshire, England) plus 20% (v/v) glycerol, and propagated in MRS broth at 30ºC before use.

Strains tested as inducers of PLNC8 production by *L. plantarum* NC8 and as indicator microorganisms are listed in Table 1. All *Lactobacillus*, *Leuconostoc*, *Pediococcus* and *Streptococcus* strains were grown in MRS broth at 30ºC, *Leuconostoc cremoris* DB1275 was grown at 25ºC, *L. acidophilus* NCDO 1748, *L. casei* strains, *L. plantarum* NCDO 1193, *L. reuteri* DSM 20016 and *L. salivarius* NCFB 2747 were grown at 37ºC, and *L. bulgaricus* ATCC 11842, *L. helveticus* ATCC 15009 and *Streptococcus thermophilus* strains were grown at 42ºC. *Enterococcus* and *Lactococcus* strains were propagated in GM17 medium, consisting of M17 medium (Oxoid) plus 1% (w/v) of glucose at 30ºC, *L. lactis* subsp. *cremoris* CNRZ 117 was grown at 25ºC, and *Enterococcus faecalis* EF1 was grown at 37ºC. *Listeria* strains, *Staphylococcus carnosus* MC1, and *Bacillus cereus* ATCC 9139 were grown in Brain Heart Infusion (BHI) medium (Oxoid) at 30, 42, and 37ºC, respectively. All these strains were maintained as frozen stocks at –80ºC in MRS, BHI, or GM17 plus 20% (v/v) glycerol and propagated twice in their corresponding broth medium before use.

Spontaneous rifampin-resistant (Rif^r, 30 µg/ml) derivatives of *L. lactis* MG1363 and *P. pentosaceus* FBB63, were isolated by sequential selection on MRS agar containing

1 increasing concentrations of the antibiotic in the range of 0.5 to 30 µg/ml (Sigma Chemical
2 Co., St. Louis, Mo.).
3
4 Bacteriocin assays
5
6 The agar drop diffusion test was used for detection of antimicrobial activity in MRS broth
7 cultures (Jiménez-Díaz et al. 1993). Lawns of each indicator strain (Table 1) were prepared
8 by inoculating ca. 10^5 cells in 4 ml of the respective soft-overlay (0.75% agar) medium
9 which was poured onto the surface of the respective agar plates.
10 The antimicrobial activity of PLNC8 was quantified in a microtiter plate assay system
11 (Geis et al. 1983), and *L. plantarum* 128/2 [PLNC8 sensitive (PLNC8^S)] was used as the
12 indicator strain (Jiménez-Díaz et al. 1995; Maldonado et al. 2003). One bacteriocin unit
13 (BU) was arbitrarily defined as the amount of bacteriocin that inhibited the growth of the
14 indicator strain by 50% (50% of the turbidity of the control culture without bacteriocin).
15 This was expressed as the reciprocal of the highest dilution exhibiting 50% inhibition of
16 the indicator strain per millilitre (BU/ml).
17
18 Testing *L. plantarum* NC8 for PLNC8 production
19
20 *Lactobacillus plantarum* NC8 MRS broth cultures were tested for PLNC8 production at
21 different phases of growth (lag-, exponential-, or stationary-phase), sizes of initial
22 inoculum (ranging from 10^2 to 10^8 CFU/ml), pH values (in the range of 4.0 to 8.0), and
23 growth temperatures (ranging from 25 to 35ºC). In all cases, culture samples were
24 withdrawn at different phases of growth, centrifuged at 10,000 × *g* for 10 min at 4ºC,
25 adjusted to pH 7.0 with 5 M NaOH, and filter-sterilized through a 0.22-µm-pore-size

1 Millex-GV filter (Millipore Iberica, S.A., Madrid, Spain). Ten μl of these cell-free
2 supernatants were spotted onto indicator lawns of *L. plantarum* 128/2. The plates were
3 incubated at the appropriate temperature for 16 h, and examined for inhibition zones.
4 Production of PLNC8 by *L. plantarum* NC8 in co-culture with the Gram-positive
5 bacteria listed in Table 1 was determined as described in Figure 1A: fresh MRS broth was
6 inoculated with 1-2% of an overnight culture of *L. plantarum* NC8 plus 0.5% of an
7 overnight culture of each strain to test for induction. The mixed cultures were held at 30ºC
8 for 6-8 h, centrifuged, and the supernatants adjusted to pH 7.0 with 5 M NaOH, filter-
9 sterilized, and finally their inhibitory activity assayed by the agar drop diffusion test, using
10 *L. plantarum* 128/2 as the indicator strain. In addition, four of these cell-free supernatants
11 showing inhibitory activity against *L. plantarum* 128/2 were selected to further determine
12 their antimicrobial spectrum against all of the strains listed in Table 1 (see footnote *c* in
13 Table 1). In control assays, all of the strains used in the mixed cultures were propagated as
14 pure cultures in their respective media and then assayed for antimicrobial activity as
15 described above.
16
17 Determination of the number of inducer cells necessary to promote PLNC8 production by
18 *L. plantarum* NC8
19
20 In order to estimate the number of inducer cells which were necessary to induce PLNC8
21 production in *L. plantarum* NC8, a fixed inoculum of the strain NC8 (10^8 CFU/ml) was co-
22 cultured in MRS broth with inocula of increasing sizes (ranging from 10^1 to 10^7 CFU/ml)
23 of the inducer strains. The mixed cultures were incubated at 30ºC for 6-8 h, and the
24 antimicrobial activity in the cell-free supernatants was quantified as described above.
25
26

Time course of PLNC8 production in co-cultures

The time course of induction of PLNC8 production by *L. plantarum* NC8 in co-culture with PLNC8-resistant (PLNC8^{R}) or PLNC8-sensitive (PLNC8^{S}) but PLNC8-inducer strains was investigated. For this, fresh MRS broth was inoculated with ca. 10^{8} CFU/ml of *L. plantarum* NC8 plus ca. 10^{7} CFU/ml of either of the inducer strains *L. lactis* MG1363 Rif^{r} or *P. pentosaceous* FBB63 Rif^{r}. The co-cultures were incubated at 30ºC, and samples were removed at different time intervals. Viable cells were differentially enumerated by spreading the previously diluted samples with a Spiral Systèm (Interscience, Saint-Nom-La Bretèche, France) on MRS agar (for *L. plantarum* NC8) or MRS agar containing 30 µg/ml of rifampin (Sigma) (for *L. lactis* MG1363 or *P. pentosaceous* FBB63). Finally, PLNC8 activity in the samples was quantified as described above.

Partial characterization of the PLNC8 inducer

Overnight broth cultures of the inducer strains (Table 1) were divided in two aliquots: one of them was centrifuged (10,000 × *g*, 10 min, 4ºC), and filtered through a 0.22-µm-pore-size Millex-GV filter (Millipore); the other part was incubated at 55ºC for 1 h or autoclaved (121ºC) for 15 min. Aliquots (50 µl) of the various cell-free supernatants or heat-treated cultures were added to fresh MRS broth containing ca. 10^{8} CFU/ml of an overnight culture of *L. plantarum* NC8. The mixtures were incubated at 30ºC for 6-8 h, and the PLNC8 activity quantified as described above. As controls, overnight broth pure cultures of the inducer strains were used for induction of PLNC8 production in *L. plantarum* NC8.

1 To test for sensitivity of the inducer to proteolytic enzymes, the heat-treated cultures
2 showing inducing activity were adjusted to pH 7.5 with 5 M NaOH, and then treated with
3 proteinase K (Sigma) at 1 U/ml (final concentration). Mixtures were incubated at 37ºC for
4 1 h, and then heated at 100ºC for 10 min to stop the reaction. Finally, the samples were
5 examined for induction of PLNC8 as described above. As a control, proteinase K was
6 inactivated by heating at 100ºC for 10 min prior to be added to the heat-treated inducer
7 cultures.

8 Finally, MRS broth was placed inside a dialysis tube (MW cutoff of 14.0 kDa) and then
9 inoculated with ca. 10^7 CFU/ml of either of the inducer strains *L. lactis* MG1363 or *P.*
10 *pentosaceous* FBB63. Each dialysis tubing was submerged into an Erlenmeyer flask
11 containing MRS broth previously inoculated with ca. 10^8 CFU/ml of *L. plantarum* NC8
12 and then incubated at 30ºC for 6-8 h. Cell-free supernatants from both separated cultures
13 were tested for PLNC8 activity.
14

15 Partial characterization of the PLNC8 autoinducer
16

17 Cell-free supernatants from *L. lactis* MG1363- or *P. pentosaceous* FBB63-*L. plantarum*
18 NC8 co-cultures (Fig. 1A) showing PLNC8 activity were tested for their ability to induce
19 bacteriocin production in *L. plantarum* NC8 as described in Figure 1B. For this, 20 μl of
20 each cell-free supernatant was added to fresh MRS broth (1 ml) containing ca. 10^8 CFU/ml
21 of an overnigth culture of *L. plantarum* NC8 and incubated at 30ºC for 6-8 h (Fig. 1B).
22 Bacteriocin activity in the cell-free supernatants (named CFS_1, Fig. 1B) was quantified as
23 described above and expressed as autoinducing units (AIU) per ml (AIU/ml). One AIU
24 was arbitrarily defined as the amount of autoinducer in the cell-free supernatants, called
25 hereafter PLNC8-autoinducer, which promoted production by *L. plantarum* NC8 of one

1 BU of PLNC8. This was expressed as the reciprocal of the highest dilution exhibiting
2 induction of PLNC8 production enough for 50% inhibition of the indicator strain per ml
3 (AIU/ml). These supernatants were further used for induction of PLNC8 production in
4 fresh *L. plantarum* NC8 cultures as described above (Fig. 1B).
5 To test for heat sensitivity of the inducer, samples (1 ml) of cell-free supernatants
6 containing 1,280 BU/ml of PLNC8 were incubated at 100ºC for intervals up to 60 min, or
7 autoclaved (121ºC) for 15 min, and the remaining activity was quantified. Sensitivity to
8 proteinase K of the PLNC8 autoinducer was carried out as described above, and the
9 remaining AIU/ml was quantified. In addition, supernatants containing autoinducing
10 activity were also placed inside a dialysis tube and then processed as described above.
11 Finally, in order to investigate whether or not PLNC8 itself was involved in the
12 autoinducing activity, the purified peptides PLNC8α and PLNC8β generated from PLNC8
13 by C_2-C_{18} reverse-phase chromatography (Maldonado et al. 2003) or mixtures of them
14 were used in autoinduction assays. For this, 0.01 μM of PLNC8α, 0.16 μM of PLNC8β or
15 mixtures of both peptides (0.01 μM of PLNC8α plus 0.16 μM of PLNC8β) were added to
16 fresh MRS broth (1 ml) containing ca. 10^8 CFU/ml of *L. plantarum* NC8, incubated at
17 30ºC for 8 h, and then the bacteriocin quantified. As a control, both individual and mixed
18 peptides were assayed by the drop diffusion test for inhibitory activity against *L. plantarum*
19 128/2.
20

1 **Results**

2

3 Induction of PLNC8 in *L. plantarum* NC8 co-cultures

4

5 The influence of pH (Ahn and Stiles 1990; Barefoot et al. 1994b; Jiménez-Díaz et al. 1993;
6 Kaiser and Montville 1993; Kleerebezem et al. 1997), temperature (Diep et al. 2000; Leal-
7 Sánchez et al. 2002; Mørtvedt-Abildgaard et al. 1995; Tichaczeck et al. 1992), and growth
8 conditions (Biswas et al. 1991; de Vuyst et al. 1996; Gutowski-Ecked et al. 1994; Jiménez-
9 Díaz et al. 1993; Leal-Sánchez et al. 2002), previously described as enhancers of
10 bacteriocin production by LAB, was studied. None of these factors appeared to promote
11 production of PLNC8 by *L. plantarum* NC8 growing alone. However, when *L. plantarum*
12 NC8 was co-cultured with several gram-positive strains, production of PLNC8 was
13 induced by forty-one out of eighty-two strains tested (Table 1). These strains belonged to
14 various genera, including *Bacillus*, *Enterococcus, Lactobacillus, Lactococcus,*
15 *Leuconostoc, Listeria, Pediococcus, Staphylococcus* and *Streptococcus*, and none of them
16 exhibited any antimicrobial activity when they were cultivated as single cultures.

17 The inhibitory spectrum of the cell-free supernatants from four different co-cultures
18 was found to be the same regardless of the identity of the strain used to induce bacteriocin
19 production by *L. plantarum* NC8 (Table 1). This could indicate that most probably the
20 same bacteriocin, PLNC8, was produced in all cases, for this bacteriocin was indeed
21 purified in a previous work after induction with two of these strains (Maldonado et al.
22 2003). On the other hand, the inducer and sensitivity phenotypes are not linked, for the
23 four types of phenotypic combinations could be observed among the strains tested (Table
24 1). Only 6% of the strains tested were able to induce PLNC8 production but they were
25 sensitive to PLNC8, and included representatives from various species belonging to the

Lactobacillus or *Pediococcus* genera. Although none of the *L. plantarum* strains were able to induce production of PLNC8, most of them were sensitive to PLNC8 (80%).

Time course of PLNC8 production by *L. plantarum* NC8 in selected co-cultures

The time courses of bacteriocin production as well as its effect on the inducer population are shown in Figure 2. Although different in total amount, the shape of the curve showing PLNC8 production was very similar in both co-cultures (Fig. 2B and 2D, respectively). Thus, the PLNC8 activity could be detected in the supernatants after 3 h of co-culture with either inducer strain, with a maximum reached after 8 (Fig. 2B) or 5 h (Fig. 2D) of incubation, respectively.

As shown in Figure 2A and 2C, the growth of the *L. plantarum* NC8 population was not affected by the co-culture with the inducer strains. Also, the *L. lactis* MG1363 population was not substantially affected by the co-culture with *L. plantarum* NC8, although a decrease in the cell number (CFU/ml) by two orders of magnitude was observed after 24 h (Fig. 2A). However, the cell number of the FBB63 strain was shown to decrease early when the PLNC8 activity was detected in the culture medium, and declined by about four log units after 24 h of incubation (Fig. 2C). These differences are most probably due to the PLNC8^S phenotype of *P. pentosaceus* FBB63.

Determination of the number of inducer cells necessary to promote PLNC8 production by *L. plantarum* NC8

A minimum inoculum of 10^4 CFU/ml of *L. lactis* MG1363 was necessary to induce PLNC8 production by *L. plantarum* NC8 (40 BU/ml). The bacteriocin production increased up to a maximum (2,560 BU/ml, for 10^6 CFU/ml of this inducer strain and

1 conditions) and then remained constant when higher inoculum sizes were used. Similar
2 results were obtained with other inducing bacteria (data not shown).
3
4 Partial characterization of the PLNC8 inducer
5
6 Cell-free supernatants from single broth cultures of any of the strains listed in Table 1 were
7 not able to induce PLNC8 production by pure cultures of *L. plantarum* NC8, thus
8 suggesting that the inducing ability was not mediated by any metabolite secreted by the
9 inducer strains into the culture medium. When the strain NC8 was co-cultured with inducer
10 strains but separated by a dialysis membrane, no PLNC8 activity was detected in the *L.*
11 *plantarum* NC8 cell-free supernatants.
12 Autoclaving of the inducer cultures prior to the addition to cultures of strain NC8 did
13 not induce PLNC8 production except for the inducer strains *L. brevis* LB9, *L. sake* NCFB
14 2714, *L. lactis* MG1363, *L. lactis* MG1614, *L. lactis* CNRZ 105 or *L. lactis* CNRZ 489.
15 However, induction of PLNC8 was completely abolished in all six cases after treatment
16 with proteinase K.
17 On the other hand, some inducer strains killed by heat treament (55ºC, 1 h) retained
18 complete inducing ability. Obviously, living cells are not necessary for induction of
19 PLNC8 production by *L. plantarum* NC8.
20
21 Partial characterization of the PLNC8 autoinducer
22
23 Figure 2 shows the time course of PLNC8-autoinducer production by *L. plantarum* NC8
24 when it was co-cultured with *L. lactis* MG1363 (Fig. 2B) or *P. pentosaceous* FBB63 (Fig.
25 2D). Differences in total amount of autoinducer at each time interval can be observed. Co-

1 culture with *L. lactis* MG1363 appears to promote higher amounts of autoinducer, which
2 was virtually constant after 5 h of incubation (Fig. 2B). In contrast, *P. pentosaceous*
3 FBB63 promoted less PLNC8 autoinducer and it reached a maximum earlier (2-3 h) (Fig.
4 2D).

5 Cell-free supernatants from *L. lactis* MG1363- or *P. pentosaceous* FBB63-*L. plantarum*
6 NC8 co-cultures showing PLNC8 activity (Fig. 1A) were examined for consecutive
7 inducing activity on fresh broth cultures of *L. plantarum* NC8 (Fig. 1B). Addition of 2-5%
8 of those supernatants (Fig. 1B, 1st induction) was found to promote bacteriocin production
9 by *L. plantarum* NC8 pure cultures (Fig. 3). Further additions of cell-free supernatants
10 from these newly PLNC8-containing *L. plantarum* NC8 cultures (Fig. 1B, CFS_2) to other
11 *L. plantarum* NC8 pure cultures resulted also in induction of bacteriocin production.
12 Nevertheless, bacteriocin activity in these consecutive supernatants decreased (from 640 in
13 CFS_1 to 80 BU/ml in CFS_2), and was completely lost in the third culture (0 BU/ml in
14 CFS_3). On the other hand, the autoinducing activity of the primary, exogenously induced
15 PLNC8-containing cell-free supernatants (640 AIU/ml in CFS) substantially decreased
16 after the first culture (80 AIU/ml in CFS_1), and was finally lost after the second one (0
17 AIU/ml in CFS_2) (Fig. 3). On the other hand, when increasing amounts of these PLNC8-
18 containing cell-free supernatants (CFS in Fig. 1A) were added to *L. plantarum* NC8 pure
19 cultures they promoted increasing amounts of PLNC8 in the new autoinduced *L.*
20 *plantarum* NC8 culture supernatants, up to a maximum of 640 BU/ml. In summary, both
21 the bacteriocin and autoinducing activities obtained upon induction of *L. plantarum* NC8
22 with cell-free supernatants from co-cultures (in the absence of inducer strains) were much
23 lower than those obtained after induction with the inducer strains themselves.

24 The PLNC8-autoinducing activity in the cell-free supernatants from *L. lactis* MG1363-
25 or *P. pentosaceous* FBB63-*L. plantarum* NC8 co-cultures was resistant to heat, being

1 completely stable even after autoclaving (Fig. 4). Nevertheless, it was sensitive to
2 proteinase K, thus indicating its proteinaceous nature. In contrast, PLNC8 activity was
3 partially lost after the heat treatments (Fig. 4). On the other hand, neither purified PLNC8α
4 or PLNC8β peptides nor mixtures of them were able to induce production of PLNC8 by *L.*
5 *plantarum* NC8 in the absence of inducer bacteria.

6 Finally, when the strain NC8 was grown in flasks in which dialysis tubing containing
7 co-culture cell-free supernatants showing autoinducing activity were placed, PLNC8
8 activity was detected in the *L. plantarum* NC8 cell-free supernatant.

9

1 ## Discussion

2

3 In this report, *L. plantarum* NC8 was shown to be unable to produce PLNC8 if grown
4 alone in liquid cultures under the environmental conditions tested. However, co-culture
5 with several Gram-positive bacteria resulted in production of PLNC8. Induction by such
6 external stimulus was not the only way for *L. plantarum* NC8 to produce bacteriocin. The
7 same stimulus also induced in strain NC8 production of a proteinaceous, heat-resistant
8 compound. That compund was shown to induce production of PLNC8 by *L. plantarum*
9 NC8 in the absence of a second, inducing bacterium. Obviously, strain NC8 possesses a
10 mechanism of autoinduction for PLNC8 production. These results indicate that most
11 probably production of bacteriocin by *L. plantarum* NC8 is a well regulated process.

12 Enhancement of bacteriocin production in LAB by co-culture with other Gram-positive
13 bacteria has been previously addressed (Barefoot et al. 1994a; Barefoot et al. 1994b; Sip et
14 al. 1998). However, in the case of *L. plantarum* NC8, bacteriocin production appeared to
15 be switched off in the absence of an inducer strain which is apparently obligatory for
16 PLNC8 production. It is presently unknown how the inducer bacteria and *L. plantarum*
17 NC8 interact to finally result in PLNC8 production. However, results presented here
18 suggest that a proteinaceous substance from the inducer strains is involved. This substance
19 did not appear to be released to the culture medium. Otherwise, cell-free supernatants or
20 dialyzed cultures from the inducer bacteria would have been able to induce production of
21 PLNC8. On the other hand, living inducer cells were not strictly necessary for induction of
22 PLNC8, for heat-killed inducer cells from some strains were able to do it. However, it is
23 not yet known whether this inducer substance is a single protein or a cell envelope
24 structure including protein components. This protein or cell envelope structure is probably
25 shared by all of the inducing strains.

1 In addition to PLNC8 production, the external stimulus provided by certain Gram-
2 positive bacteria also promotes production of a PLNC8-autoinducing activity by *L.*
3 *plantarum* NC8, here termed PLNC8-autoinducer. Based on the present findings, this
4 autoinducer can be differentiated from PLNC8. First, neither PLNC8 nor its individual
5 peptides PLNC8α or PLNC8β were able to induce PLNC8 production in *L. plantarum*
6 NC8. Second, whereas PLNC8 was sensitive to autoclaving (retained only 12.5% of the
7 initial inhibitory activity), the autoinducer activity remained unchanged (Fig. 4). And third,
8 it is known that autoinducers are effective at concentrations as low as 10^{-14} M or less (Nes
9 et al. 1996; Nes and Eijsink 1999). In our case, no autoinducing activity was detected after
10 a second induction, whereas bacteriocin activity was still detected (Fig.1B and Fig. 3).
11 In the case of *L. plantarum* NC8, it has been demonstrated that the presence of other
12 bacteria in the same environment triggers bacteriocin production. From an ecological point
13 of view, such regulatory mechanism could be part of a defence strategy which includes
14 sensing of other bacteria and a response to these by production of bacteriocin. As soon as
15 the inducing cells have disappeared from the culture medium or their population is below a
16 certain threshold, bacteriocin production can no longer be maintained. The inducing
17 bacteria also activate the production of an autoinducer molecule in *L. plantarum* NC8
18 which apparently promotes both bacteriocin and of the autoinducer itself. However, the
19 amounts of bacteriocin and autoinducer detected in that case were apparently much lower
20 than those detected after exposure to the inducing bacteria. Hence, the stimulus provided
21 by the inducer strains seems to be primarily necessary for bacteriocin production in *L.*
22 *plantarum* NC8. On the other hand, the presence of an autoinducing activity suggests the
23 existence of a quorum sensing mechanism which regulates bacteriocin production the
24 population of *L. plantarum* NC8. Actually, the presence of two direct repeats upstream of
25 the putative –35 region of *plNC8*B, the gene encoding PLNC8β (Maldonado et al. 2003)

1 which agrees with the consensus sequence and structure of promoters of class II
2 bacteriocins regulated by autoinduction suggests a similar mechanism.
3

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2

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Table 1. Induction of plantaricin NC8 (PLNC8) production by *L. plantarum* NC8 in co-culture with gram-positive bacterial strains, and inhibitory spectrum of the induced bacteriocin.

Bacterial species	Source[a]	Induction of PLNC8 production in strain NC8[b]	Sensitivity to induced PLNC8 from co-culture CFSs[c]
Bacillus cereus ATCC 9139	TNO	+	R
Enterococcus faecalis EF1	TNO	+	R
E. faecalis JH2- 2	INRA	-	R
E. faecalis OG1-X	UG	+	R
E. faecalis BM 4100 WT	TNO	+	R
E. faecalis CNRZ 135	INRA	+	R
E. faecalis CNRZ 136	INRA	+	R
E. faecalis CNRZ 137	INRA	+	R
E. faecium LP6T1a	Our collection	+	R
E. faecium LP6T1a-20[d]	Our collection	+	R
Lactobacillus acidophilus ATCC 4356	TNO	-	R
L. acidophilus NCDO 1748	NCDO	+	R
L. brevis LB9	UV	+	R
L. bulgaricus ATCC 11842	TNO	+	R
L. casei ATCC 334	TNO	-	R
L. casei NCDO 161	NCDO	+	R
L. casei NCDO 393	CIT	-	R
L. curvatus NCFB 2739	TNO	-	R
L. fermentum ATCC 9338	TNO	+	S
L. fermentum ATCC 14933	ATCC	-	S
L. fermentum NCDO 1750	NCDO	+	S
L. helveticus ATCC 15009	TNO	+	R
L. hilgardii LB76	UV	+	S
L. pentosus ATCC 8041	CECT	-	S
L. plantarum ATCC 8014	ATCC	-	S
L. plantarum BOM1	Our collection	-	S
L. plantarum LB6	UV	-	S
L. plantarum LPC1	Our collection	-	S
L. plantarum LPC13	Our collection	-	S
L. plantarum LPC2	Our collection	-	S
L. plantarum LPD1	Our collection	-	S
L. plantarum LPD5	Our collection	-	S
L. plantarum LPD7	Our collection	-	S
L. plantarum LPE1	Our collection	-	R
L. plantarum LPE5	Our collection	-	S
L. plantarum LPP1	Our collection	-	S

Table 1. continued (a)

Bacterial species	Source[a]	Induction of PLNC8 production in strain NC8[b]	Sensitivity to induced PLNC8 from co-culture CFSs[c]
L. plantarum LPP5	Our collection	-	R
L. plantarum LPS1	Our collection	-	S
L. plantarum LPS5	Our collection	-	S
L. plantarum LPS21	Our collection	-	S
L. plantarum LPS26	Our collection	-	S
L. plantarum LPS29	Our collection	-	S
L. plantarum LPT 70/3	Our collection	-	R
L. plantarum NCDO 1193	CIT	-	R
L. plantarum 37A	Our collection	-	S
L. plantarum 55-1[d]	Our collection	-	R
L. plantarum 15N/1	Our collection	-	S
L. plantarum 128/2	Our collection	-	S
L. plantarum 144/1	Our collection	-	S
L. reuteri DSM 20016	TNO	+	R
L. sake NCFB 2714	TNO	+	S
L. salivarius NCFB 2747	TNO	+	R
Lactococcus lactis IL1441	INRA	+	R
L. lactis IL1403	INRA	+	R
L. lactis MG1363	CIT	+	R
L. lactis MG1614	CIT	+	R
L. lactis subsp. *cremoris* CNRZ 105	INRA	+	R
L. lactis subsp. *cremoris* CNRZ 106	INRA	+	R
L. lactis subsp. *cremoris* CNRZ 113	INRA	+	R
L. lactis subsp. *cremoris* CNRZ 114	INRA	+	R
L. lactis subsp. *cremoris* CNRZ 117	INRA	+	R
L. lactis subsp. *cremoris* CNRZ 158	INRA	+	R
L. lactis subsp. *cremoris* CNRZ 163	INRA	+	R
L. lactis subsp. *cremoris* CNRZ 485	INRA	+	S
L. lactis subsp. *cremoris* CNRZ 489	INRA	+	R
L. lactis subsp. *cremoris* CNRZ 511	INRA	+	R
Leuconostoc cremoris DB1275	TNO	-	R
L. mesenteroides 32	INRA	+	R
L. mesenteroides 33	INRA	+	R
Listeria innocua BL 86/26	TNO	+	R
L. monocytogenes NCTC 7973	FVM	-	R
L. monocytogenes LI5 sv1/2	FVMN	-	R
L. monocytogenes NCTC 5105	FVM	-	R

Table 1. continued (b)

Bacterial species	Source[a]	Induction of PLNC8 production in strain NC8[b]	Sensitivity to induced PLNC8 from co-culture CFSs[c]
L. monocytogenes LI1 sv4	FVM	-	R
L. monocytogenes Scott A	FVM	-	R
Pediococcus damnosus NCDO 1832	CIT	-	R
P. parvulus P339	UV	-	R
P. pentosaceous FBB63	TNO	+	S
P. pentosaceous PC1	TNO	+	R
P. pentosaceous P56	UV	-	R
Staphylococcus carnosus MC1	TNO	+	R
Streptococcus thermophilus ST20	TNO	+	R
S. thermophilus ST112	TNO	+	R

[a]Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); CECT, Colección Española de Cultivos Tipo (Universidad de Valencia, Burjasot, Spain); CIT, Cranfield Institute of Technology (United Kingdom); DSM, DMSZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany); FVM, Facultad de Veterinaria, Universidad Complutense (Madrid, Spain); INRA, Institut National de la Recherche Agronomique (Jouy-en-Josas, France); NCDO, National Collection of Dairy Organisms (Reading, United Kingdom); NCFB, National Collection of Food Bacteria, c/o NCIMB Ltd. (Aberdeen, Scotland, United Kingdom); NCTC, National Collection of Type Cultures (Central Public Health Laboratory, London, United Kingdom); TNO, Nutrition and Food Research (Zeist, The Netherlands); UT, Universität Tübingen (Tübingen, Germany); UV, Universidad de Valencia (Burjasot, Spain); UG, Universidad de Granada (Granada, Spain).

[b]PLNC8 determined in cell-free supernatants (CFSs) from mixed cultures of *L. plantarum* NC8 plus the tested strain was assayed by the agar drop diffusion test (Jiménez-Díaz et al. 1993) with *L. plantarum* 128/2 as the indicator strain (Maldonado et al. 2003). Symbols: +, induction of PLNC8; -, not induction.

[c]Sensitivity of the listed species to the PLNC8 produced by *L. plantarum* NC8 when it was co-cultured either with *L. lactis* MG1363, *P. pentosaceous* FBB63, *L. salivarius* NCFB 2747, or *L. mesenteroides* 33. Symbols: S, sensitive; R, resistant.

[d]Non-bacteriocin producing strains derived from bacteriocin producing ones: *E. faecium* LP6T1a-20 from the enterocin I producer *E. faecium* LP6T1a (Floriano et al. 1998), and *L. plantarum* 55-1 from the plantaricins S and T producer *L. plantarum* LPCO10 (Jiménez-Díaz et al. 1993).

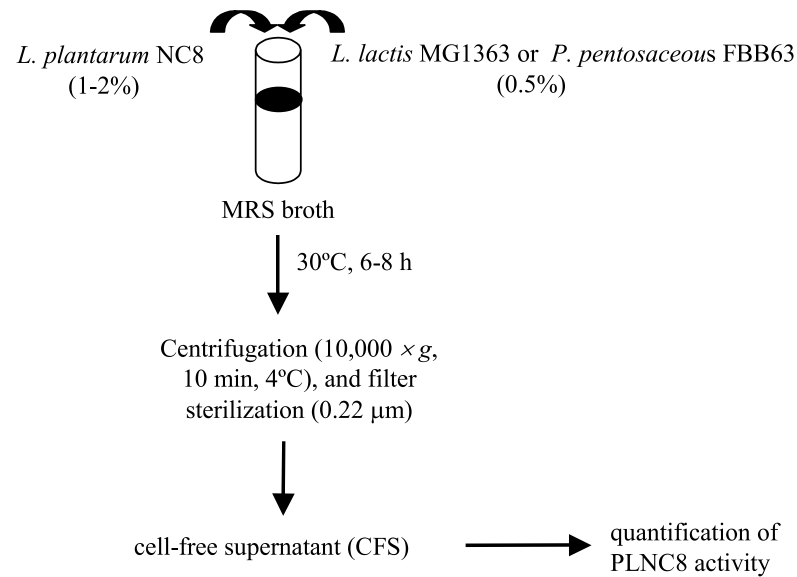
Legends of the Figures

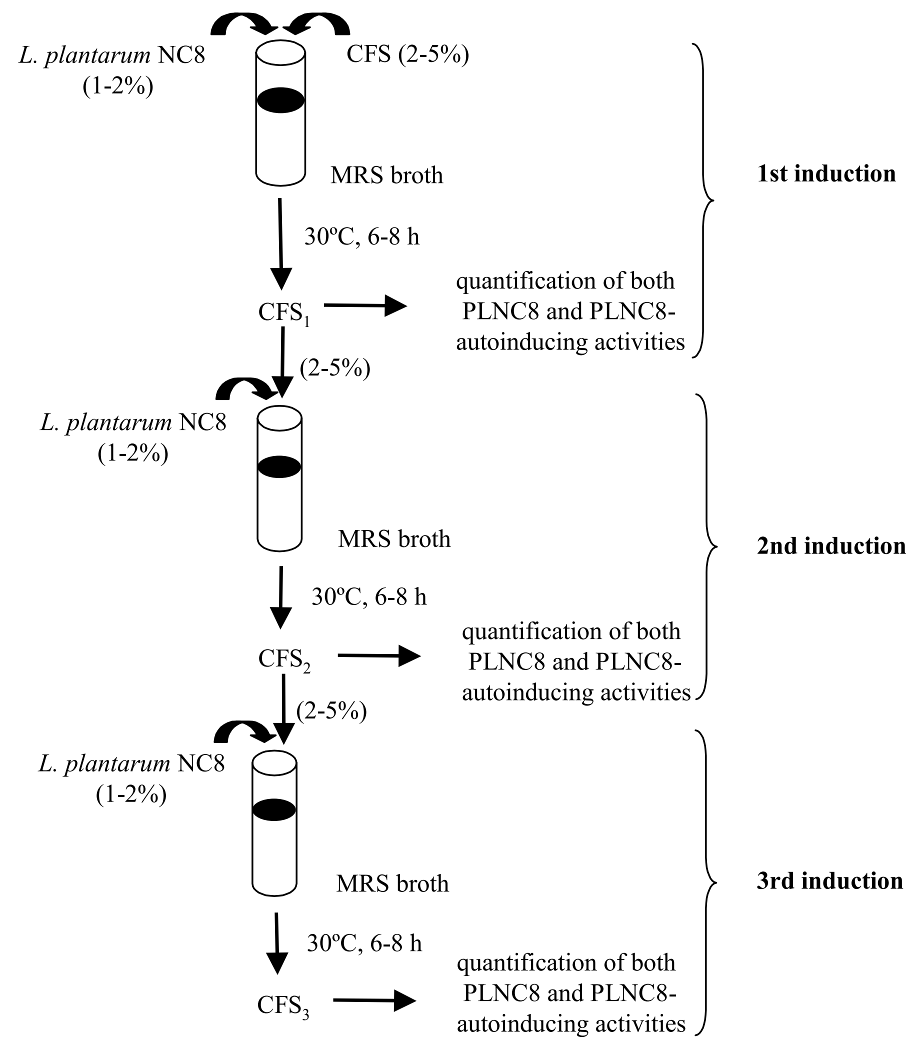
Figure 1. The procedure for induction of PLNC8 production by *L. plantarum* NC8. Percentages (v/v) in parentheses indicate proportion of inoculum or cell-free supernatant in total (final) volume.

Figure 2. Growth (log CFU/ml), induced PLNC8 activity (log BU/ml), and PLNC8-autoinducing activity (log AIU/ml) in *L. plantarum* NC8-*L. lactis* MG1363 (panels A and B) or *L. plantarum* NC8-*P. pentosaceous* FBB63 co-cultures (panels C and D). Symbols: in A and C, *L. plantarum* NC8 in single (●) and in co-cultures (○); *L. lactis* MG1363 in single (▲) and in co-cultures (△); and *P. pentosaceous* FBB63 in single (◆) and in co-cultures (◇). In B and D, PLNC8 (■) and PLNC8-autoinducing activities (□) in the cell-free supernatants.

Figure 3. PLNC8 and PLNC8-autoinducing activities in consecutive cultures of *L. plantarum* NC8. CFS, cell-free supernatant from co-culture of *L. plantarum* NC8 and *L. lactis* MG1363; CFS_1, *L. plantarum* NC8 culture induced with a cell-free supernatant from CFS; and CFS_2, *L. plantarum* NC8 culture induced with a cell-free supernatant from CFS_1. Neither PLNC8 nor PLNC8-autoinducing activities were detected in CFS_3 (see Figure 1B).

Figure 4. Sensitivity to heat of co-culture induced *L. plantarum* NC8 cell-free supernatants showing PLNC8 activity.


A) Induction of PLNC8 production by co-culture
L. plantarum NC8
(1-2%)
L. lactis MG1363 or P. pentosaceous FBB63
(0.5%)
MRS broth
30ºC, 6-8 h
Centrifugation (10,000 × g, 10 min, 4ºC), and filter sterilization (0.22 µm)
cell-free supernatant (CFS)
quantification of PLNC8 activity


B) Induction of PLNC8 production by cell-free supernatants
L. plantarum NC8
(1-2%)
CFS (2-5%)
MRS broth
30ºC, 6-8 h
CFS1
quantification of both PLNC8 and PLNC8-autoinducing activities
1st induction
(2-5%)
L. plantarum NC8
(1-2%)
MRS broth
30ºC, 6-8 h
CFS2
quantification of both PLNC8 and PLNC8-autoinducing activities
2nd induction
(2-5%)
L. plantarum NC8
(1-2%)
MRS broth
30ºC, 6-8 h
CFS3
quantification of both PLNC8 and PLNC8-autoinducing activities
3rd induction

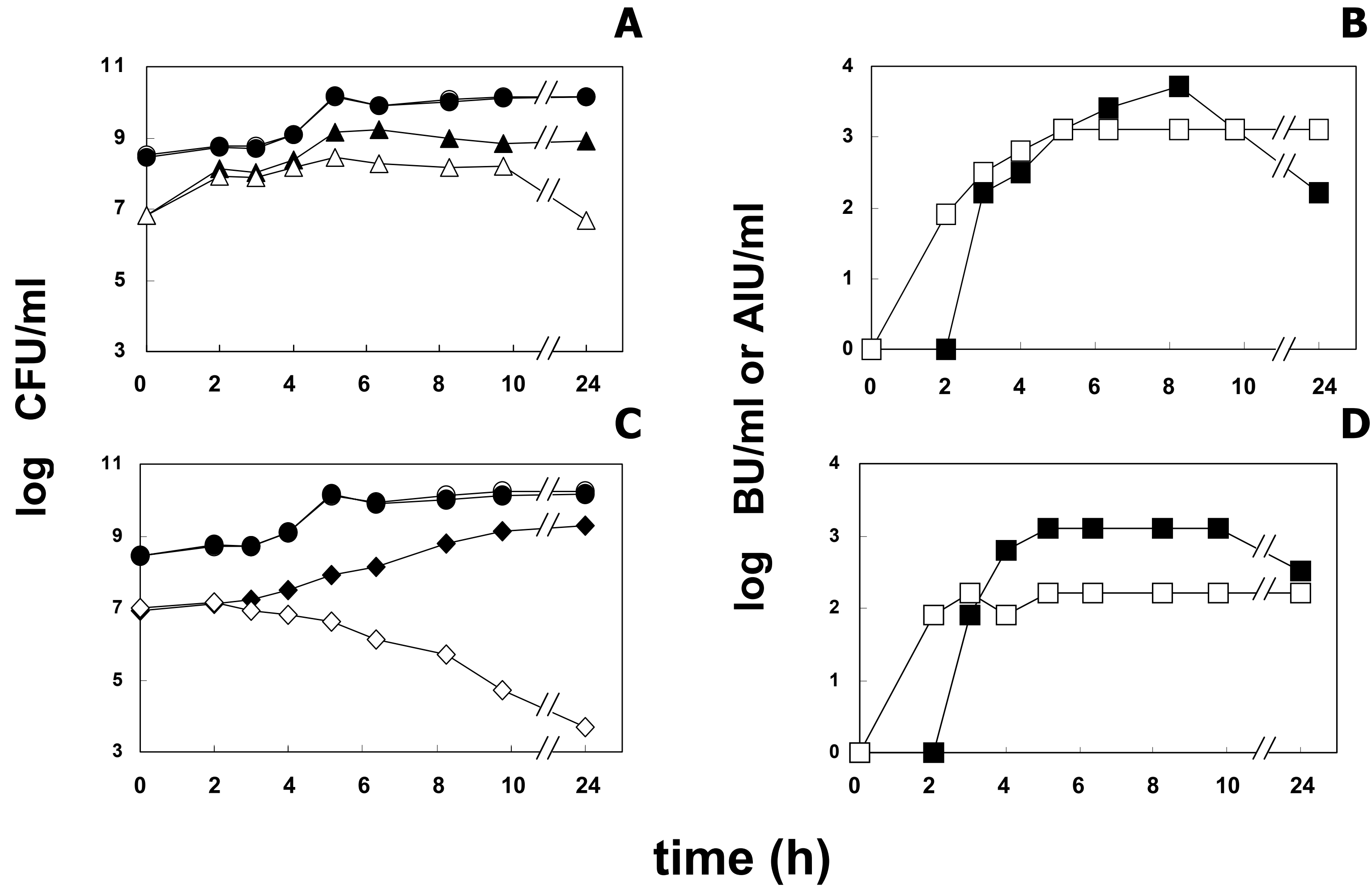


Figure 2

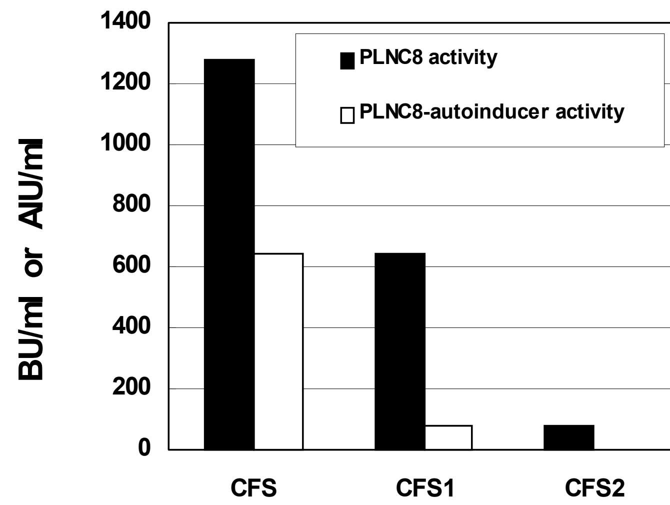


Figure 3

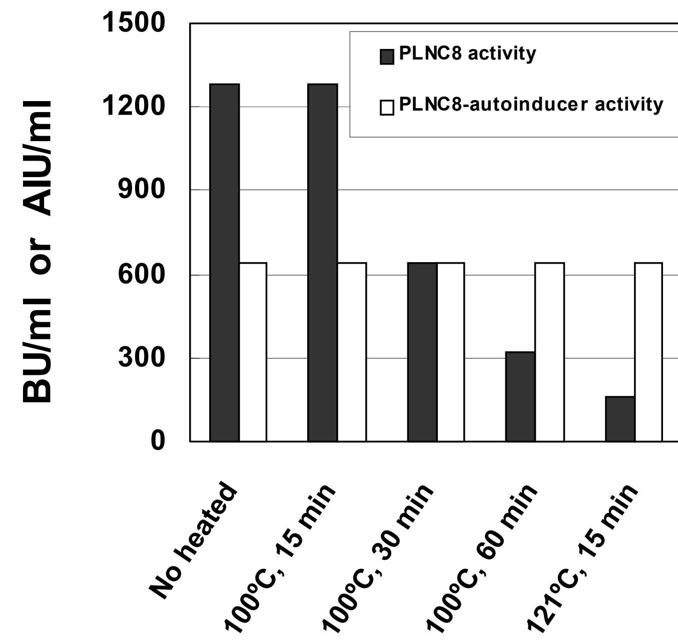


Figure 4